THE EPIDEMIOLOGY AND NATURAL HISTORY OF GENITAL HERPES SIMPLEX VIRUS (HSV) INFECTION

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by

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DECLARATION OF ORIGINAL AUTHORSHIP

'Declaration

'I, [Meghna Ramaswamy] confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

DEDICATION

This thesis is dedicated to the memory of my late father, Mr. V. N. Ramaswamy (February 1940 - November 2005). Papa, I wish you could be here so we could celebrate and enjoy together the achievement that this thesis represents.

ABSTRACT

Genital infection with herpes simplex virus type 2 (HSV-2) is increasingly common worldwide. The aims of this thesis were to investigate the epidemiology and natural history of genital herpes among GUM attendees with symptomatic genital herpes, and among HIV-1 infected individuals. In our study of GUM attendees in the UK, we demonstrated HSV-1 to account for 9% of first episodes of genital herpes. These findings are in contrast with observations made elsewhere in the UK, where HSV-1 has accounted for >50% of first-episode cases. As most individuals with genital HSV-2 infection remain clinically misdiagnosed, the need for improved diagnostic methods to detect genital HSV infection is warranted. We compared the performance of virus culture and PCR in patients clinically diagnosed with genital herpes. PCR increased HSV detection in patients with both early and late presentations and in first and recurrent diseases. PEG precipitation was the most sensitive specimen preparation method of choice. A HSV positive PCR was also associated with heterosexuals, early presentation, and visible genital ulceration. HSV-1 and HSV-2 genital infections were also associated with white and black ethnicity respectively. This suggests that host susceptibility and behavioural factors may influence the epidemiological patterns of genital herpes. Current data support the use of HSV type-specific serology and PCR to diagnose HSV infections. In addition to PCR, we evaluated the performance of type-specific serology (HerpeSelect EIA, Focus Technologies, Cypress, California, USA) for the diagnosis of HSV infection. Our findings indicated that increasing the assay cut-off from 1.1 to 3.1 increased specificity and maintained sensitivity. By performing an inhibition EIA using an inhibition value of

≥60% we minimised false positive results from Ugandan and Kenyan sera. Recent data has implicated HSV-2 as a co-factor in HIV transmission. We therefore investigated the seroepidemiology of HSV infection among 850 HIV-infected individuals. HSV-2 seroprevalence was 63% and increased with age and was associated with female gender, heterosexuals and black ethnicity. A follow-up of 123 HSV-2 seronegative persons revealed a HSV-2 seroconversion rate of 10% which was associated with HPV infection and gonorrhoea. Only 21% of the seroconverters received a clinical diagnosis of genital herpes, and this was more likely in persons diagnosed HIV-1 positive before 1997 (pre-HAART era). To investigate the effects of HAART on HSV-specific immunity, we studied the kinetics of HAART-induced reconstitution of HSV-specific T-cell responses in HIV-1 infected persons at different stages of clinical disease using an ELISPOT assay. Successful HAART proved to resolve HSV-specific immunity restoration which coincided with HAART-induced CD4 gain. HSV-specific IFN-γ responses correlated with CD4 counts. Responses similar to those of HIV-negative healthy controls were seen at CD4 counts >450 cells/mm^3.

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LIST OF ABBREVIATIONS

ABC	Abacavir
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
Ala	Alanine
ALP	Alkaline phosphatase
AMEN	AIDS in Multiethnic Neighbourhoods
APC	Antigen presentation cell
Arg	Arginine
ATP	Adenosine triphosphate
ATV	Atazanavir
AZT	Zidovudine
BA	Black-African
BC	Black-Caribbean
BCA	Bicinchoninic acid
bp	Base pair
BSA	Bovine serum albumin
CPE	Cytopathic effect
CMV	Cytomegalovirus
Cp	Crossing point
CSF	Cerebrospinal fluid
CSW	Commercial sex worker
DC	Dendritic cell
DDI	Didanosine

DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotide triphosphate
dUTP	Deoxyuridine triphosphate
dTTP	Deoxythymidine triphosphate
EBV	Epstein Barr virus
EFV	Efavirenz
EIA	Enzyme immunoassay
ELISPOT	Enzyme linked immunospot assay
EM	Erythema multiforme
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FCS	Foetal calf serum
FDA	Food and drug administration
FPV	Fosamprenavir
FRET	Fluorescence resonance energy transfer
FSW	Female sex worker
FTC	Emtricitabine
gB	Glycoprotein B
gC	glycoprotein C
gD	Glycoprotein D
gG	Glycoprotein G
gH	Glycoprotein H
gL	Glycoprotein L
gp120	Glycoprotein 120

GSK	GlaxoSmithKline
GUD	Genital ulcer disease
GUM	Genitourinay medicine
HAART	Highly active antiretroviral therapy
HAEM	Herpes simplex virus associated erythema multiforme
HHV-6	Human herpes virus-6
HHV-7	Human herpes virus-7
HHV-8	Human herpes virus-8
HIV-1	Human immunodeficiency virus type 1
HPA	Health Protection Agency
HPV	Human papilloma virus
HSE	Herpes simplex virus encephalitis
Hsp	Heat shock protein
HSPG	Heparan sulfate chains on proteoglycans
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVE	Herpes virus entry proteins
IB	Immunoblot
IE	Immediate early
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalactopyranoside

IRD	Immune restoration disease
ISC	Indian sub-continent
IVDU	Intravenous drug user
KSHV	Kaposi's sarcoma- associated herpes virus
LAT	Latency associated transcript
LC	LightCycler
LPV	Lopinavir
LTNP	Long term non progressor
LTR	Long terminal repeat
LTSP	Long term slow progressor
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
MHC	Major histocompatability complex
MSM	Men who have sex with men
NaCl	Sodium chloride
Nef	Negative factor
NHANES	National Health and Nutrition Survey
NK	Natural killer cell
NNRTI	Non-nucleoside transcriptase inhibitor
NRTI	Nucleoside transcriptase inhibitor
NSy	Non-synonymous
NVP	Nevaripine
OD	Optical density
ORI	Origin of replication
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PHA	Phytohemagglutinin
PI	Protease inhibitor
PMSF	Phenylmethylsulfonylfluoride
r	Ritonavir
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RTV	Ritonavir
SEB	Staphylococcal enterotoxin B
SFC	Spot forming cells
SI	Single insertion
SQV	Saquinavir
SS	Single substitution
STI	Sexually transmitted infection
STD	Sexually transmitted disease
Sy	Synonymous
TAP	Transporter associated with antigen presentation
Tat	Transactivator
TDF	Tenofovir disoproxil fumarate
TG	Trigeminal ganglia
Th	T-helper cells
TK	Thymidine kinase
Tm	Melting temperature

TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TPV	Tipranavir
UK	United Kingdom
UL	Unique long region
US	Unique short region
USA	United States of America
VC	Virus culture
VERO	African green monkey kidney cells
Vhs	Virion host shutoff
VP	Virion protein
Vpu	Viral protein U
VTM	Virus transport medium
VZV	Varicella zoster virus
W	White
3TC	Lamivudine
D4T	Stavudine

LIST OF PUBLICATIONS

This thesis is based on the following original papers and manuscripts, which will be referred to in the text.

Ramaswamy, M., Sabin C, McDonald C., Smith, M., Taylor, C., and Geretti, A. M. 2006. Herpes simplex virus type-2 (HSV-2) seroprevalence at the time of HIV-1 diagnosis and seroincidence after HIV-1 diagnosis in an ethnically diverse cohort of HIV-1 infected persons. *Sexually Transmitted Diseases.* 33:96-101.

Ramaswamy, M., Waters, A., Smith, C., Hainsworth E., Hardy, G., Johnson, M., Ainsworth, J., Phillips, A., and Geretti, A. M. Reconstitution of Herpes Simplex Virus (HSV)-specific T-cell immunity in HIV-1 infected patients receiving highly active antiretroviral therapy (HAART). *Journal of Infectious Diseases. In press.*

Ramaswamy, M., and Geretti, A. M. Interactions and management issues in HSV and HIV co-infection. *Expert Review of Anti-infective Therapy. In press.*

Paz-Bailey, G., **Ramaswamy, M.,** Hawkes, S., and Geretti, A. M. Genital Herpes: Epidemiology and management options in resource-poor settings. *Sexually Transmitted Infections. In press.*

Nebbia, G., Mattes, F., **Ramaswamy, M.**, Quaglia, A., Verghese, G., Griffiths, P. D., Burroughs, A., and Geretti, A. M. 2005. Primary herpes simplex virus type-2 (HSV-2)

infection as a cause of liver failure after liver transplantation. *Transplant Infectious Disease. In press.*

Ramaswamy, M., Smith, M., Thomas, D., Maxwell, S., and Geretti, A. M. 2005. Detection and typing of herpes simplex DNA in genital swabs by real-time polymerase chain reaction. *Journal of Virological Methods.* 126:203-206.

Ramaswamy M., McDonald, C., Sabin, C., Tenant-Flowers, M., Smith, M., and Geretti, A. M. 2005. The epidemiology of genital infection with herpes simplex virus type 1 and type 2 in genitourinary medicine attendees in inner London. *Sexually Transmitted Infections.* 81:306-308.

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Ramaswamy, M., Bowden, R., Smith, M., and Geretti, A. M. Sequence analysis of the herpes simplex virus type 2 UL14 gene in clinical isolates from an ethnically diverse population with genital herpes. *Manuscript in preparation.*

1. Chapter 1. Main Introduction

There are 8 types of human herpes viruses (Table 1.1). Among the alpha herpesviruses, two biologically distinct subtypes of herpes simplex virus (HSV) exist. One third of the world's population has been shown to be infected with either subtype (HSV-1 and HSV-2) (Whitley *et al.,* 1998). HSV is the main cause of genital ulcer disease (GUD) in the developed world and also accounts for a significant proportion of GUD cases in developing countries (Serwadda *et al.,* 2003; Bruisten *et al.,* 2003). HSV-1 is traditionally associated with orofacial infections but can also cause genital infections. HSV-2 is associated with genital infections. In the USA, 22% of individuals aged 12 years or over are HSV-2 seropositive (Fleming *et al.,* 1997). In the developing world, HSV-2 prevalence ranges between 60-90% (Narouz *et al.,* 2002).

HSV infection is life-long. Following primary infection either by direct contact with mucosal surfaces or abraded skin, HSV migrates from the nerve endings to the local sensory or autonomic ganglia, where it establishes a latent state (Yeung-Yue *et al.,* 2002). The establishment of latency is a common feature of the herpesvirus family to evade the human immune response. Reactivation of the virus occurs commonly and can be triggered by local or systemic stimuli such as immunodeficiency, trauma, fever, menstruation, ultra-violet rays, and sexual intercourse (Kirchner *et al.,* 2000). Upon reactivation, infective virions travel down the sensory axons to replicate in the epithelial cells of the skin or mucosa at or near the site of original infection. Genital herpes is of public health importance due to its morbidity, psychological impact, frequency of recurrence and rare but serious neonatal damage which may occur from

maternal transmission of HSV (Brugha *et al.,* 1997). Clinical presentations of genital herpes may be as a first or a recurrent episode. The infection is classified as a primary, initial or non-primary and recurrent (Brugha *et al.,* 1997). Primary infections occur in persons lacking immunity to both HSV types. Non-primary infection may occur in individuals with previous exposure to one HSV type. HSV infections are often asymptomatic and present with mild symptoms. The virus can be shed and transmitted in the absence of recognised disease and male to female transmission is more efficient for HSV- (Corey & Handsfield, 2000; Mertz *et al.,* 1992).

HSV-2 is prevalence is high among individuals infected with human immunodeficiency virus-1 (HIV-1). The strong epidemiological association between HIV-1 and HSV-2 may reflect common risk factors for infection. In addition, there is also evidence for a more direct biological interaction between the two viruses. Prospective studies have shown HSV-2 to increase the risk of HIV-1 acquisition and transmission by approximately two-fold (Blower *et al.,* 2004; Celum *et al.,* 2004). Genital ulceration due to HSV-2 is thought to increase HIV-1 transmission by increasing the levels of HIV-1 shedding through genital lesions, and by providing a portal of entry for the virus (Mbopi-Keou *et al.,* 2003). There is also evidence to show plausible biological interactions between HIV-1 and HSV-2, with HIV-1 increasing the clinical expression of HSV-2 (Holmberg *et al.,* 1988). In individuals with cellular immunodeficiency caused by HIV-1, HSV-2 reactivation is associated with greater frequency of HSV-2 shedding and increased HIV-1 viremia (Fauci, 1988; Posavad *et al.,* 2004). Studies are currently underway to determine whether the use of anti-herpetic agents such as acyclovir, can reduce the risk of HIV-1 transmission. A

greater understanding of the natural history of HSV-2 infection is required to understand the possible role it plays in the dissemination and acquisition of HIV-1.

Table 1.1. The different members of the herpes family

Herpes virus type	Family
Herpes simplex virus type 1 (HSV-1)	Alpha
Herpes simplex virus type 2 (HSV-2)	Alpha
Varicella zoster virus (VZV)	Alpha
Cytomegalovirus (CMV)	Beta
Human herpes virus -6 (HHV-6)	Beta
Human herpes virus-7 (HHV-7)	Beta
Epstein-barr virus (EBV)	Gamma
Human herpes virus-8 (HHV-8) Kaposi's sarcoma- associated herpes virus (KSHV)	Gamma

1.1 THE BIOLOGY OF HSV-2

1.1.1 Virion characteristics

HSV-1 and HSV-2 are members of the sub-family α-herpes viruses (Figure 1.1). The virion has a distinct large virion structure (150-200nm) comprising of a core containing linear double-stranded DNA, an icosahedral capsid containing 162 hexagonal capsomers surrounded by an amorphous tegument containing additional viral proteins, and an outer membrane envelope studded with viral glycoproteins.

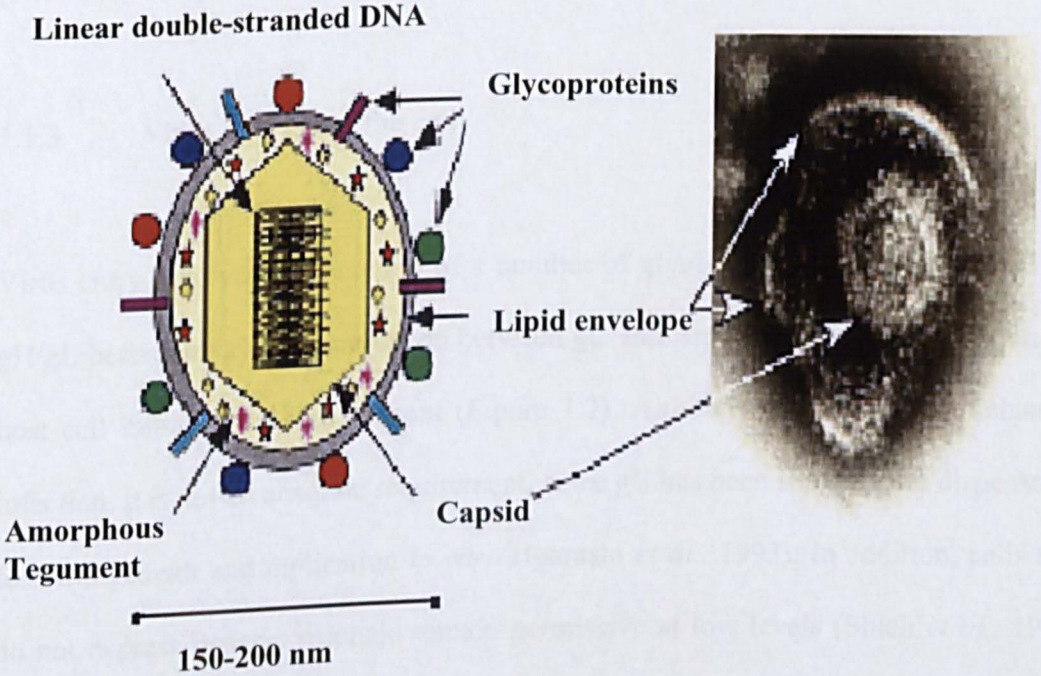


Figure 1.1. HSV virion structure

The virion has four major components: Double-stranded DNA, the icosahedral capsid shell, the viral tegument and the lipid membrane envelope with glycoprotein spikes.

1.1.2 Genome Organisation

The HSV genome consists of approximately 150,000 base-pairs, encoding at least 80 gene products. The genome consists of two covalently linked segments called Long (L) and short (S) based upon their relative length. Each segment contains unique regions of sequences (UL or US) which are flanked with inverted repeat sequences. Some viral genes and their products are named by their relative position from left to right in the UL or US region and some are alternatively named upon their function. The genome also consists of other elements which orchestrate replication and processing and packaging of newly synthesised genomes.

1.1.3 Virus entry into the cell

Virus entry involves the function of a number of glycoproteins (gB, gC, gD and the gH/gL heterodimer). An interaction between gC and heparin sulphate moieties on the host cell initiates virus attachment (Figure 1.2). Although this initial step enhances infection, it is not an absolute requirement, since gC has been shown to be dispensable for viral growth and replication *in vitro* (Igarashi *et al.,* 1993). In addition, cells that do not express heparin sulphate remain permissive at low levels (Shieh *et al.,* 1992; WuDunn *et al.,* 1989). Virion attachment is stabilised by interactions of the gD protein with recently identified cellular receptors referred to as 'herpes virus entry' proteins (HVE). Fusion of the viral envelope with the cellular membrane occurs through an undetermined mechanism involving proteins gD, gB and the gH/gL heterodimer. Fusion results in the nucleocapsid and some tegument proteins being

released into the cytoplasm of the cell. Fusion results in the nucleocapsid and some tegument proteins being released into the cytoplasm of the host cell. The nucleocapsid and some of the tegument proteins get transported via microtubules into the nucleus for the initiation of viral replication while other tegument proteins remain in the cytoplasm.

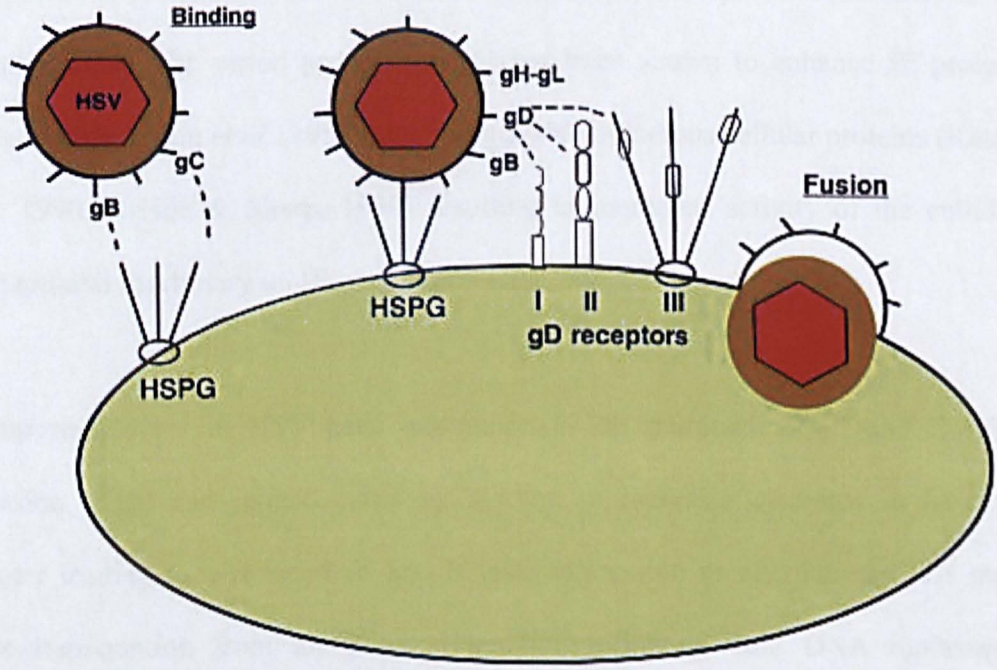


Figure 1.2. HSV entry into cells

Binding of HSV to heparan sulphate chains on cell surface proteoglycans (HSPG) initiates cell contact. Viral glycoproteins gB or gC can mediate binding. HSV gD can bind to entry receptors, including HVE (I), a member of the TNF-receptor family; nectin-1 or nectin-2; (II) two related members of the immunoglobulin superfamily or, (III) sites generated in heparan sulfate by the action of specific 3-*O*-sulfotransferases. Binding of gD to its receptors activates viral fusion.

1.1.4 Virus replication

HSV genes are expressed in 3 classes: immediate early (IE or α), early (E or β) and late (L or γ) (Figure 1.3a). Immediate early genes are transcribed immediately after infection and activate transcription of early genes. Early gene products replicate viral DNA, which in turn stimulates the expression of late genes. The initiation of IE gene transcription is followed by the recruitment of cellular transcriptional machinery to IE gene promoters. The virion protein (VP16) has been shown to enhance IE protein expression (Batterson *et al.,* 1983) and interact with numerous cellular proteins (Katan *et al.,* 1990; Kristie & Sharp, 1990) resulting in increased activity of the cellular transcriptional machinery on IE gene promoters.

An important event in HSV gene expression is the shutdown of IE and E gene expression. ICP4 can repress itself by binding to repressor elements in its own promoter leading to a reduced IE and E gene expression or alternatively IC8 may reduce transcription from the parental genome following viral DNA replication (Godowski & Knipe, 1985). In addition, HSV may shut down host transcription, replication and translation (Roizman & Tognon, 1983). For instance, the virion associated protein (VHS) degrades mRNA early in infection (Kwong *et al.,* 1988) and ICP22 modifies the binding ability of the host RNA polymerase II complex following infection (Spencer *et al.,* 1997).

DNA replication occurs within replication compartments of infected cells and both viral DNA and replication proteins are localised to cellular structures called ND10 sites. Replication compartments form at these sites as viral DNA synthesis proceeds.

The linear HSV DNA circularises upon entry into the nucleus and replication is initiated at one of the three origins of replication (OriL or OriS) located in the UL or US region of the genome (Figure 1.3b). Seven genes essential for viral replication have been identified (UL9, UL29, UL5, UL52, UL8, UL30, UL42) which encode a single-stranded DNA binding protein, a helicase-primase complex, and a DNA polymerase. In addition, 'non-essential' E gene products for viral DNA replication may also be expressed (e.g. Thymidine kinase). Synthesis of L gene products is required for capsid assembly, however, the mechanism of DNA cleavage and packaging is not very well understood. The route of virion egress from an infected cell remains controversial. It is thought that enveloped virions fuse with the outer nuclear membrane, releasing nucleopcapsids into the cytoplasm that re-envelope by budding into the Golgi compartment, which then get secreted from the cell in a vesicular route (Skepper *et al.*, 2001).

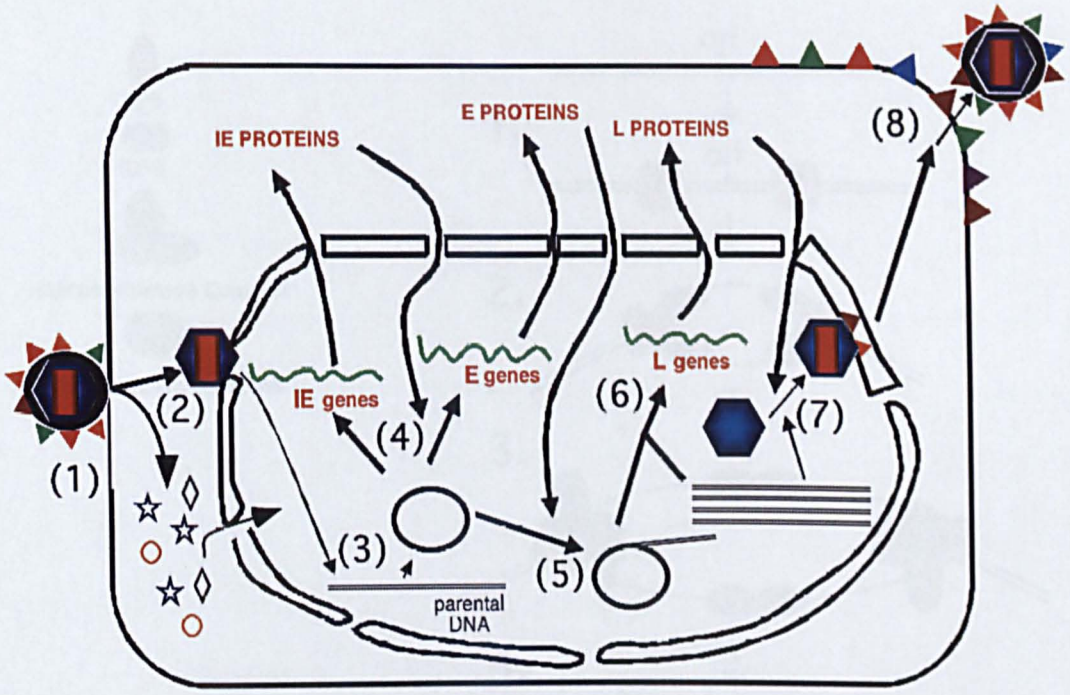


Figure 1.3a. Productive HSV replication cycle in an infected cell.

Stages of HSV infection include: (1) Receptor binding and membrane fusion; (2) Release of the viral nucleocapsid and tegument in the cytoplasm of the cell and nucleocapsid transport to the nuclear pore; (3) Release of the viral DNA into the nucleus; (4) Transcription and translation of viral immediate early (IE) and early (E) genes; (5) Viral DNA synthesis; (6) Transcription and translation of viral late (L) genes; (7) capsid assembly and DNA packaging; and (8) Egress of progeny virions (Adapted from Taylor *et al.*, 2002).

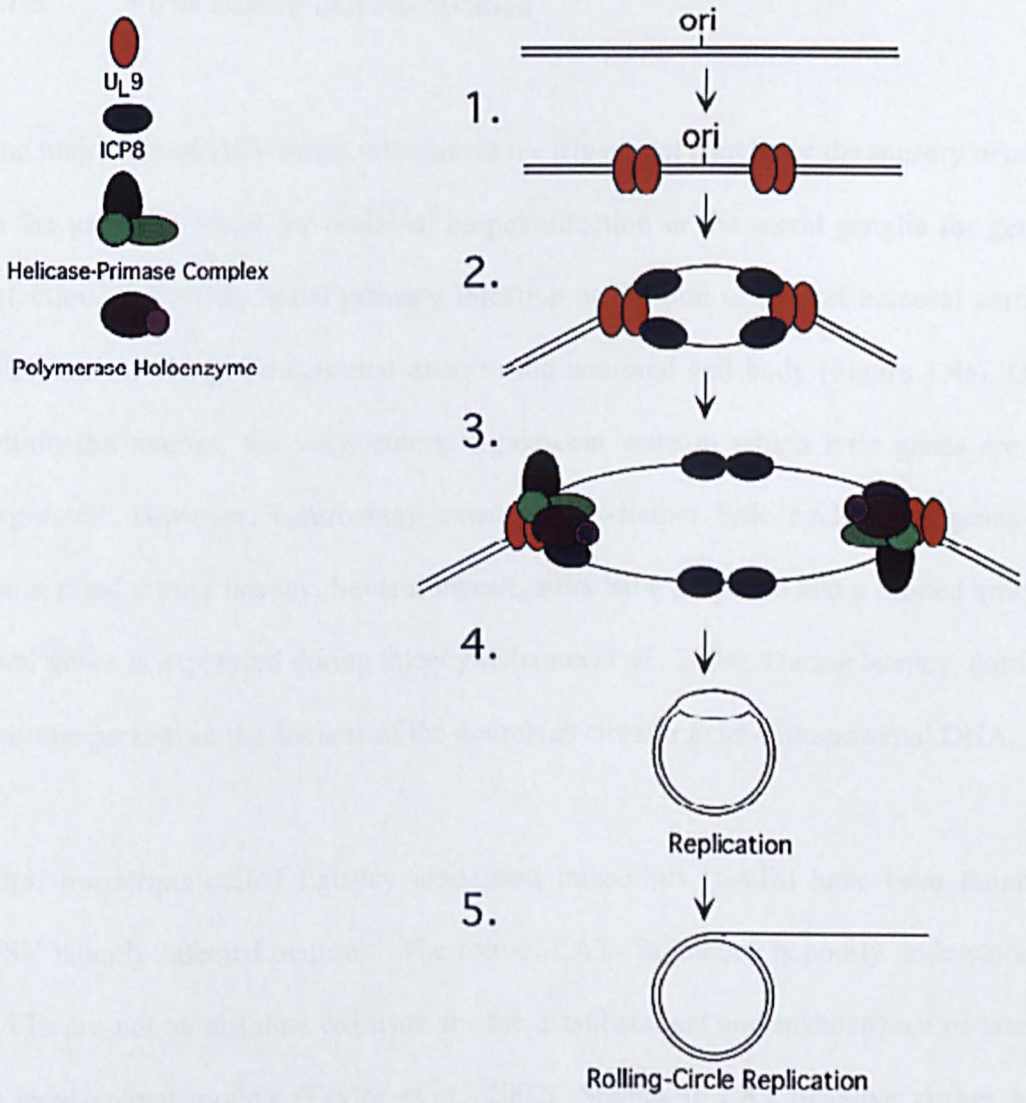


Figure 1.3b. HSV DNA replication

Stages of HSV replication are: (1) UL9, the origin binding protein, binds to specific sites at origin (ori L or ori S) and begins to unwind the virus DNA; (2) The single-stranded DNA binding protein, ICP8, is recruited to the unwound DNA; (3) UL9 and ICP8 recruit the five remaining replication proteins to the replication forks; (4) DNA replication proceeds via a theta replication mechanism; and (5) switches to a rolling-circle replication mechanism (Adapted from Taylor *et al.*, 2002).

1.1.5 Virus latency and reactivation

The major site of HSV latent infection is the trigeminal ganglia or the sensory neurons in the ganglion tissue for orolabial herpes infection or the sacral ganglia for genital infection. Following initial primary infection at the oral or genital mucosal surface, HSV travels along the neuronal axon to the neuronal cell body (Figure 1.4a). Once within the neuron, the virus enters a quiescent state in which lytic genes are not expressed. However, controversy exists over whether lytic cycle viral genes are transcribed during latency. Several investigators have proposed that a limited array of viral genes is expressed during latency (Khanna *et al.*, 2004). During latency, the viral genome persists in the nucleus of the neuron as circular extra-chromosomal DNA.

Viral transcripts called Latency associated transcripts (LATs) have been found in HSV latently infected neurons. The role of LATs in latency is poorly understood as LATs are not an absolute requisite for the establishment and maintenance of latency in most animal models (Taylor *et al.*, 2002). Studies in LAT negative viruses have demonstrated increased immediate early gene expression in neurons, suggesting LATs to limit viral gene expression and promote a latent state (Garber *et al.*, 1997). In addition, LATs may protect neurons from apoptosis allowing neuronal survival during latent infection (Perng *et al.*, 2000). HSV transcriptional silence during latency allows the virus to evade immune surveillance, until signals are generated that cause virus reactivation. Upon reactivation, new progeny virions are generated, resulting in the virus travelling through the neuron to the site of primary infection to re-initiate the lytic cycle (Figure 1.4b).

The effectiveness of the immune response to primary HSV-1 infection may determine the likelihood of a reactivation event. The observation that lymphocytes and their cytokine products persist in latently infected trigeminal ganglia of mice suggest a possible continuing role for the immune system in controlling HSV-1 recurrences (Liu *et al.,* 2000). Several studies have demonstrated the presence of $CD8^+$ T cells for prolonged periods in the trigeminal ganglia (TG) after HSV-1 infection, and have shown these cells to surround neurons following *in vivo* induced reactivation of HSV-1 from latency (Cantin *et al.,* 1995; Liu *et al.,* 2000; Liu *et al.,* 1996; Shimeld *et al.,* 1995; Shimeld *et al.,* 1996). In one study, $CD8^+$ T cells present in the trigeminal ganglia 14 days after HSV-1 corneal infection could completely block HSV-1 reactivation from latency for at least 2 weeks in explanted TG cultures (Liu *et al.,* 2000). In addition, IFN-γ has also shown to inhibit HSV-1 reactivation, ICP0 promoter activity, gC promoter activity in latently infected neurons (Decman *et al.,* 2005; Halford *et al.,* 2001).

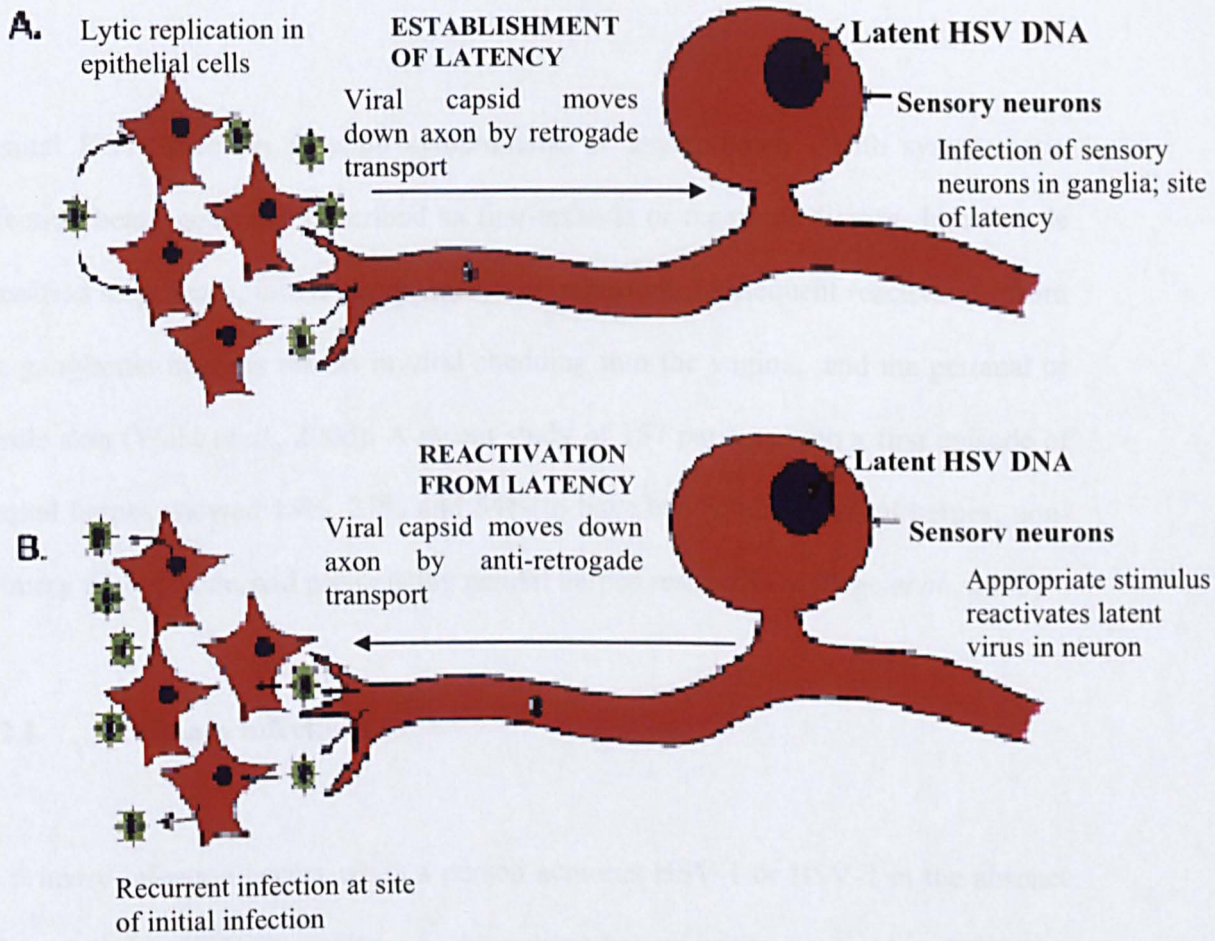


Figure 1.4a and 1.4b. HSV Latency and Reactivation

Stages of HSV latency are as follows: (a) HSV replicates in epithelial cells or the genital mucosa, enters the sensory neurons and travels up the axon to the neuronal cell body, where it establishes a latent state; and (b) Upon appropriate stimulus, the virus can reactivate, travel back down the axon and re-establish lytic replication (Adapted from Taylor *et al.,* 2002).

1.2 THE NATURAL HISTORY OF HSV-2 INFECTION

Genital HSV infection may be symptomatic or asymptomatic, with symptomatic infection being generally described as first-episode or recurrent disease. Infection is classified as primary, initial non-primary and recurrent. Subsequent reactivation from the ganglionic neurons results in viral shedding into the vagina, and the perianal or penile skin (Wald *et al.,* 2000). A recent study of 157 patients with a first episode of genital herpes showed 18%, 27% and 54% to have had primary genital herpes, non-primary first episode, and pre-existing genital herpes respectively (Page *et al.,* 2003).

1.2.1 Primary infections

A primary infection occurs when a person acquires HSV-1 or HSV-2 in the absence of pre-existing HSV immunity, i.e. the patient has no antibodies against HSV-1 or HSV-2. Symptomatic HSV-2 primary infections are characterised by genital lesions, pain, itching, dysuria, vaginal or urethral discharge and systemic symptoms. The lesions are usually painful, and may last for a mean of 16.5 days in men and 19.7 days in women (Corey *et al.,* 1983). The lesions begin as small vesicles, get ulcerated and may sometimes crust. The virus is shed from the infected area for a median of 11 days following the onset of lesions, and may last for more than 30 days in some patients. Prospective studies of HSV-2 serodiscordant couples have shown that most individuals seroconvert to HSV-2 in the absence of clinical symptoms (Wald *et al.,* 2001; Corey *et al.,* 2004). Primary infections that are initially silent may become symptomatic months or years later.

1.2.2 Initial infections

Initial or non-primary infections are newly acquired infections with one HSV type, in the presence of pre-existing antibodies to another HSV type. The development of IgG antibodies following HSV infection varies from 21 to over 42 days with most individuals having detectable IgG 21–28 days after exposure to infection and probably persisting for life (Ashley *et al.,* 1988; Slomka *et al.,* 1995; Ho *et al.,* 1993). IgM antibodies are usually detectable 9–10 days after exposure and last 7–14 days, and may remain detectable for up to 6 weeks in a minority of individuals (Ho *et al.,* 1992; Ashley, 1998).

1.2.3 Recurrent infections

The clinical symptoms of recurrent genital herpes are less severe and of shorter duration than the primary episode. Recurrences may be symptomatic or asymptomatic. Symptomatic recurrences are characterised by the presence of small vesicular ulcers which last for a mean of 10.6 days in men and 9.3 days in women (Corey *et al.,* 1983). For patients who experience a first episode of genital herpes, the risk of recurrent disease is higher among those infected with HSV-2 than HSV-1 (Engelberg *et al.,* 2003; Wald *et al.,* 2000). In the first twelve months after a first episode of genital herpes, the risk of a symptomatic recurrence is 89% with HSV-2 and 50-57% with HSV-1 (Benedetti *et al.,* 1994; Corey *et al.,* 1983; Langenberg *et al.,* 1999). Recurrence rates of genital herpes are more frequent in men than women, with median monthly recurrence rates reported to be 0.43 in men and 0.33 in women (Benedetti *et al.,* 1994). Earlier studies have reported HSV recurrences to decrease

over time (Knox *et al.,* 1982; Catotti *et al.,* 1993). In a study of 664 persons diagnosed with genital herpes and followed up for 14 months median recurrence rates within the first 12 months of follow up were 1 for HSV-1 and 5 for HSV-2. Significantly lower rates of recurrences were evident in this population after 12 months. Among patients with HSV-2 recurrent disease, a decrease in the number of recurrences was strongly associated with longer follow up (Benedetti *et al.,* 1999). These findings are in contrast with observations made elsewhere which showed HSV recurrence rates to not decline over time (12 months) in 457 patients (Benedetti *et al.,* 1994). This could be accounted by potential population bias, whereby the authors reported half of the patient population to fail to complete the full year of follow-up.

1.2.4 Atypical manifestations and unrecognised infection

The clinical manifestations of genital herpes are diverse, with non-classical symptoms leading to misdiagnoses of infection. These include herpetic whitlow, erythema multiforme, eczema herpeticum and less commonly, gingivostomatitis. The variability in clinical symptoms of genital herpes means that identification of HSV-2 infection on clinical grounds alone is insufficient and that laboratory assays must be utilised for accurate diagnosis and effective clinical management of patients with genital herpes.

Typical symptoms of primary genital HSV-2 infection include genital lesions, pain, itching, dysuria, vaginal or urethral discharge and systemic symptoms. The infection can be confused with other genital conditions, leading to four out of five people with genital HSV-2 infection remaining un-diagnosed (Corey, 1994). Among HSV-2 infected individuals 60% of infections may be unrecognized, 20% may be

asymptomatic and the remaining 20% being symptomatic recognized infection. A study of 779 female STD clinic attendees, of whom 372 were HSV-2 seropositive showed only 22% of women who were HSV-2 antibody positive reported symptoms (Koutsky *et al.,* 1992).

1.2.5 Asymptomatic shedding and HSV transmission

Asymptomatic infection has been implicated in the person-person transmission of HSV-2 infection (Mertz *et al.,* 1985; Barton *et al.,* 1987). Asymptomatic shedding occurs when the virus is present on the mucosal or skin surface of an individual in the absence of clinical symptoms, and is most common during the period before and after a clinical recurrence. In a prospective study of 144 discordant couples, where one partner had recurrent genital herpes, and the other had no serological or clinical evidence of HSV-2 infection, the risk of HSV-2 transmission during asymptomatic shedding was 69% (Mertz *et al.,* 1992). In another study involving 27 women with recurrent genital herpes, asymptomatic shedding was detected in 80% of those followed up for more than 50 days, with the virus being shed asymptomatically overall on 1% of the days (Brock *et al.,* 1990). To evaluate the temporal relation between asymptomatic and symptomatic episodes, Wald et al (1995) calculated the rate of asymptomatic shedding within seven days of a symptomatic recurrence. Results demonstrated that 30% of asymptomatic shedding episodes occur 7 days preceding an episode and 20% occurs in the 7 days after an episode (Wald *et al.,* 1995).

The time since HSV acquisition may also influence the rate of asymptomatic shedding. As it is observed for symptomatic recurrent disease, the frequency of asymptomatic shedding is significantly higher with genital HSV-2 than HSV-1 infection. A prospective study showed that asymptomatic shedding was more common in the first 12 months after an HSV-2 first-episode than an HSV-1 first episode (Koelle *et al.,* 1992). In the same study, the rate of shedding among women with primary HSV-2 infection decreased from 4.3% in the first 12 months to 2.3% after infection in the following year. Asymptomatic shedding may involve more than one genital site with rates of 0.7% and 1.1% being observed for the cervix/vulva and perianal areas (Wald *et al.,* 1995). Among MSM, the perianal area is the most common site of asymptomatic virus shedding, and among HIV-1 negative heterosexual men, the penile skin is the most common site for virus shedding (Wald, 2004). Since the natural history of HSV infection is characterised by asymptomatic shedding between outbreaks, both recognised and unrecognised infections contribute to transmission and spread.

1.3 DIAGNOSIS OF HSV INFECTION

The diagnosis of genital herpes is often challenging because its non-classical presentations are often misinterpreted by the patient and the physician. A clinical diagnosis of genital herpes has a predictive value of 40% (Koutsky *et al.,* 1990), indicating that HSV laboratory tests must be utilised to make a definitive diagnosis. The detection of HSV in cell culture has been considered the most optimal method for the diagnosis of genital herpes in the presence of genital lesions (Ashley, 1993).

Serological assays which discriminate between HSV-1 and HSV-2 type-specific proteins (gG-1 and gG-2 respectively), contribute towards the laboratory diagnosis.

1.3.1 Virus culture and immunofluorescence

Isolation of HSV in cell culture has long been considered as the 'gold standard' test (Ashley, 1993). HSV strains replicate in most cell lines within 12-18 hours and result in a cytopathic effect (CPE) within 16-24 hours following inoculation. Although specificity of the technique is nearly 100%, there are many limitations. The levels of virus shedding, quality of specimen and transport conditions influence sensitivity (Wald *et al.,* 2003; Corey *et al.,* 1983; Scoular, 2002). The rate of virus recovery declines significantly with time since the onset of lesions, from 52-93% for vesicles to 41-72% for ulcers and 19-27% for crusted lesions (Corey *et al.,* 1983; Scoular, 2002). Furthermore, lack of specimen refrigeration after collection and during transport markedly reduces virus viability (Wald *et al.,* 2003). Type-specific confirmatory assays such as immunofluorescence relying on monoclonal antibodies being directed to type-specific antigens are used to differentiate between HSV types.

1.3.2 PCR

PCR is a highly sensitive method used to directly detect and type HSV and has been the method of choice for detecting HSV infections of the CNS (Lakeman & Whitley, 1995). Among patients diagnosed with herpes simplex encephalitis, PCR is 96% sensitive and 99% specific (Boivin, 2004). Several published studies have shown the optimal genes for HSV amplification to be those coding for thymidine kinase, DNA

polymerase, DNA binding protein, gC, gG and gD (Boivin, 2004). Although earlier PCR techniques were laborious, expensive and prone to contamination, newly developed real-time PCR assays are fully automated and allow virus-specific detection within a closed system, with fast turn-around times and low risk of contamination (Espy *et al.,* 2001; Burrows *et al.,* 2002). The introduction of Light Cycler technology, a commercially available system that performs PCR with real-time detection of amplified products by fluorescence resonance energy transfer (Espy *et al.*, 2000) has allowed high speed thermal cycling and rapid detection (Kessler *et al.*, 2000). The melting curve analysis of this instrument differentiates between HSV-1 and HSV-2 types (Whiley *et al.,* 2004; Schmutzhard *et al.,* 2004; Koenig *et al.,* 2001). Due to its increased sensitivity and fast turn-around times, the recommendation of PCR to be used as a diagnostic test for HSV detection in genital swabs among asymptomatic persons warranted. It is the only suitable virologic test for testing HSV in certain clinical settings, such as the diagnosis of neonatal herpes or suspected central nervous system HSV infection (Kimberlin, 2004; Boivin, 2004).

1.3.3 Serology

At the population level, the number of diagnosed cases of genital herpes significantly under-estimates the burden of infection. HSV-2 seroprevalence data can help assess the burden although they do not account for cases caused by HSV-1. Serological testing can provide useful information in symptomatic patients when direct virus detection methods yield negative results and can identify patients with unrecognised sub-clinical disease (Ashley, 2001). The western blot has been used for nearly 15 years, and has been extensively validated. The test produces bands corresponding to

glycoprotein G (gG) and other proteins of HSV that progress in intensity and complexity during seroconversion (Ashley, 1998). Studies have documented the specificity for HSV-1 and 2 by this assay to be 100% (Ashley, 1993). The disadvantage of the western blot is that its time consuming, expensive and profiles may not always be definitive. The test is therefore unlikely to be developed for commercial use (Ashley, 2001). HSV type-specific serology is particularly useful in diagnosing HSV infection in asymptomatic individuals (Ashley & Wald, 1999; Wald, 2002). Currently STI management guidelines favour the screening for STD/HIV in selected patients, individuals engaging in high-risk behaviour or those in partnerships where the partner remains HSV-2 infected.

An alternative approach to detect HSV type-specific antibodies is by enzyme immunoassay (EIA). In previous years there was limited use for the HSV EIA as it was unable to reliably differentiate between HSV-1 and 2 antibodies due to cross-reactivity (Ashley *et al.*, 1998). Since HSV-1 and HSV-2 share significant sequence homology, cross reactivity is a major problem. Glycoprotein G (gG) (gG1 in HSV-1 and gG2 in HSV-2) is the only known viral protein to predominantly elicit a type-specific B-cell response (Wald, 2002). gG1, a 238 amino acid envelope protein of unknown function (McGeoch *et al.*, 1985), is a prototype antigen for type-specific HSV-1 serology (Lee *et al.*, 1986; Sanchez-Martinez *et al.*, 1991) and forms the basis of commercially available enzyme immunoassays (Ashley *et al.* 1998). Its counterpart, HSV-2 (gG2) is successfully used as an antigen in type-specific HSV-2 seroassays (Ho *et al.*, 1992; Lee *et al.*, 1985; Svennerholm *et al.*, 1984). Both gG1 and gG2 contain long stretches of type-unique amino acids that harbour type-specific antibody epitopes. Data has shown purified anti-gG2 antibodies to be non-reactive to

gG1 peptides and gG1 antigens (Liljeqvist *et al.,* 1998). The cleavage product of gG2 (sgG2), when evaluated in an EIA has a specificity and sensitivity of >98%. In addition, monoclonal antibodies directed against sgG2 have identified type-specific linear and nonlinear epitopes devoid of cross-reactivity to HSV-1 antigens. Type-specific IgG antibody responses have also been detected in sera from HSV-infected patients by using sgG2 as the antigen (Görander *et al.,* 2003). Commercially available kits based on gG1 and gG2 demonstrate sensitivity and specificity of 85-100% and 80-98% respectively, (Leone, 2003).

1.4 EPIDEMIOLOGY OF HSV-2 INFECTION

HSV-2 genital infection is highly prevalent worldwide (Fleming *et al.*, 1997; Halioua & Malkin, 1999; O'Farrell, 1999), although seroprevalence varies between regions and populations. Data taken from 10 developed countries (USA, Canada, Japan, Germany, UK, Belgium, France, Italy, Spain and Australia) have estimated 107 million individuals to be HSV-2 seropositive (Marchant & Roe, 1997). Of those, 86 million are estimated to have symptomatic disease, although only 21 million have a confirmed diagnosis of genital herpes. In addition, HSV-2 seropositivity rates are high among HIV-1 infected individuals, ranging from 33% in Rome, Italy (Suligoi *et al.,* 2002), 65% in Seoul, Korea (Kim *et al.,* 2003), 81% in HIV-1 positive men in Baltimore, USA (Hook *et al.,* 1992), to 95% in HIV-1 positive female commercial sex workers in Zaire, Africa (Schomogyi *et al.,* 1998). Several studies have demonstrated HSV-2 and HIV-1 to be strongly associated with each other (Kjetland *et al.,* 2005; Serwadda *et al.,* 2003; Wald, 2004; Reynolds & Quinn, 2005). Although preliminary evidence suggest that genital herpes may be implicated in the spread and

dissemination of HIV-1 in both the developing and developed world (Barton *et al.*, 2005; Ramjee *et al.*, 2005; Wald, 2004) more prospective studies are required to determine the possible risk

1.4.1 Developed world

Genital herpes is the most common infectious cause of genital ulceration in the developed world and the incidence of infection continues to rise. In the National Health and Nutrition Examination Survey (NHANES), a large ongoing population-based study in the USA, an increase in HSV-2 seroprevalence from 16% (midpoint 1978) to 22% (midpoint 1991) was observed among individuals aged 12 years and above in the general population (Fleming *et al.*, 1997). The 30% increase in HSV-2 seropositivity from 1978-1994 in the general population was associated with a shift towards a younger age of acquisition (Johnson *et al.*, 1989). Higher seroprevalences were observed among women than men (26 % vs. 18%), and black groups than hispanic or caucasian groups (46% vs. 22% vs. 18%). In Europe, the HSV-2 seroprevalence in the general population ranges from 4% in England and Wales to 24% in Bulgaria (Pebody *et al.*, 2004).

1.4.1.1 HSV-2 prevalence in the general population

Population-based studies have demonstrated HSV-2 prevalence to vary between industrialised countries (Table 1.2). In the U.S. general population, HSV-2 antibody prevalence has been reported to be 22% (Fleming *et al.*, 1997), whereas rates in the United Kingdom general population vary between 3-5% (Vyse *et al.*, 2000). One

cross-sectional study among women aged between 18-29 years in low-income neighbourhoods in California, USA, demonstrated 35% of individuals to be HSV-2 seropositive with predictors of HSV-2 infection being black ethnicity, older age, lower income, parity, greater number of lifetime male sexual partners, earlier onset of sexual intercourse, sexual favours, past history of STDs and cocaine use (Buchacz *et al.,* 2000).

Table 1.2. HSV-2 seroprevalence in non high-risk populations of the developed world

Country	Cohort	Sex	Age* (range)	n	% HSV-2+	Ref
USA						
National 1988-1994	Random sample of civilians (NHANES III)	F/M	(12-≥70)	13094	22	Fleming *et al.*, 1997
Washington 1991-1992	Family medical centre attendees	F/M	(18-45)	315/185	27/15	Brown *et al.*, 1997
California 1996-1998	Low-income neighbourhoods	F	(18-29)	1635	35	Buchacz *et al.*, 2000
UK						
London 1981-1982	Antenatal patients	F	(<20-≥35)	3533	10	Ades *et al.*, 1989
England & Wales 1994-1995	Immunocompetent individuals	F/M	(16-69)	3347	5.1/3.3	Vyse *et al.*, 2000
Canada						
British Columbia 1999	Antenatal patients	F	(15-44)	1215	17	Patrick *et al.*, 2001
Sweden						
Malmö 1990-1993	Antenatal patients	F	(15-≥36)	1190	21	Persson *et al.*, 1995
Finland						
Helsinki 1988-1989	Antenatal patients	F	(< 20-> 41)	997	16	Arvaja *et al.*, 1999
Norway						
Oslo 1992-1994	Antenatal patients	F	(NG)	960	27	Eskild *et al.*, 2000
France						
National 1996	Random sample of civilians (HERPIMAX)	F/M	(35-60)	4412	17	Malkin *et al.*, 2002
Spain						
Madrid 1993-1994	Random sample	F/M	(15-45)	692	3.5	De Ory *et al.*, 1999
New Zealand						
Dunedin 1993-1994	Random sample	F/M	21	372/407	4.3/2.7	Ebehart-Phillips *et al.*, 1998
Australia						
Sydney 1995-1998	Antenatal patients	F	28	2616	13	Mindel *et al.*, 2000
Israel	Healthy blood donors					
Tel Aviv 2003-2004		F/M	(3-79)	3677	7	Davidovici *et al.*, 2005
Japan						
Central Japan 1993	Random sample from rural towns	F/M	(20-49)	158/105	1.2/1.8	Hashido *et al.*, 1999

*Age expressed as mean/median in years unless otherwise stated; F, female; M, male

1.4.1.2 HSV-2 prevalence among high-risk populations

Several serological surveys have been conducted among high risk populations (Table 1.3). Among STD clinic attendees in the Netherlands, HSV-2 seropositivity rates range from 32% in Amsterdam (Van de Laar *et al.,* 1998) to 22% in Rotterdam (Roest *et al.,* 2001), although only 20% of HSV-2 seropositive individuals had a history of genital herpes. In a population of STD clinic attendees in Paris, France 55% of the population was HSV-2 seropositive, with older age and female gender being predictors of infection (Janier *et al.,* 1999). Studies carried out among STD clinic attendees in Rome, Italy and Madrid, Gijon and Malaga, Spain, have reported 27% and 25% to be seropositive for HSV-2 respectively (Cusini *et al.,* 2000; Varela *et al.,* 2001). Findings from Australian serological surveys have shown 65% of heterosexual men attending an STD clinic in Sydney, Australia to be HSV-2 seropositive. This is an unusual finding as it is the highest recorded prevalence for HSV-2 infection among heterosexual men in the developed world (Bassett *et al.,* 1994; Malkin, 2004). In a cross-sectional study among STD clinic attendees in the USA without any history of genital herpes, 26% were HSV-2 seropositive. Higher rates observed in women, black ethnicity and in those aged 30 years or over ($P<0.01$) (Whittington *et al.,* 2001).

Factors that may contribute to the increased rates of HSV-2 infection include increasing numbers of sexual partners and failure of safe sexual practices. A reduction in the prevalence of prior HSV-1 infection among youths has also been proposed. There remains limited evidence to suggest that HSV-1 seropositivity provides protection against HSV-2 infection. Defining roles of HSV-1 acquisition in childhood

may contribute to the increasing number of diagnoses of genital herpes as pre-existing HSV-1 immunity has shown to attenuate the clinical expression of HSV-2 upon infection.

Table 1.3. HSV-2 seroprevalence in high-risk populations in the developed world

Country	Cohort	Sex	Age* (range)	N	% HSV-2+	Ref
USA						
Washington 1984-1986	STD clinic attendees	F	24	776	43	Koutsky *et al.*, 1992
Washington 1989-1995	HIV-1 infected women	F	27	60	75	Hitti *et al.*, 1997
Washington 1998	STD clinic attendees	F/M	(≤19-≥40)	606	26	Whittington *et al.*, 2001
UK						
Central London 1992	Heterosexual STD clinic attendees	F/M	(17-69)	347/294	25/17	Cowan *et al.*, 1994
	Homosexuals	M	(19-69)	192	27	
France						
Paris	STD clinic attendees	F/M	(NG)	487	55	Janier *et al.*, 1999
Northern France, Paris (NG)	HIV-1 infected individuals	F/M	(<25->45)	534	59	Andréoletti *et al.*, 2005
Netherlands						
Rotterdam 1998	STD clinic attendees	F/M	(13-71)	653	22	Roest *et al.*, 2001
Amsterdam 1986-1988	STD clinic attendees	F/M	(<20-≥40)	1679	32	Van de Laar *et al.*, 1998
Germany						
Jena 1996-1997	HIV-1 infected individuals	F/M	(20-39)	110/272	66/40	Wutzler *et al.*, 2000
Italy						
Rome 1986	STD clinic attendees	F/M	(18≥53)	941	41	Mele *et al.*, 1988
Northern Italy 1997-1998	STD clinic attendees	F/M	(18-75)	919	27	Cusini *et al.*, 2000
Spain						
Madrid 1996-1997	STD clinic attendees	F/M	(18-65)	374	25	Varela *et al.*, 2001
Australia						
Auckland 1991-1992	STD clinic attendees	F/M	(<20-≥50)	123/171	30/22	Perkins *et al.*, 1996
Sydney (NG)	Homosexuals	M	(NG)	194	65	Bassett *et al.*, 1994
Japan						
Osaka 1985-1989	Commercial sex workers	F	35	70	80	Hashido *et al.*, 1998

*Age expressed as mean/median in years unless otherwise stated; (NG) data not available; F, female; M, male

1.4.2 HSV-2 seroprevalence in the UK

In the UK, HSV-2 prevalence ranges from 4-10% in the general population from 16-69 years, with a trend of increasing seropositivity with age for both sexes (Vyse *et al.*, 2000; Morris-Cunnington *et al.*, 2004). However the above studies did not include large numbers of samples from adults in London. Further studies are required to determine the burden of HSV-2 infection in the general population of London. In selected populations in the UK, HSV-2 prevalence rates of 8% and 23% have been recorded among blood donors and STD clinic attendees respectively in London, with higher rates in homosexuals and females (Cowan *et al.*, 1994). This might be because females aged between 17-68 years comprised a large proportion of the cohort (42%) compared to heterosexuals (35%) or homosexuals (23%). In another study among STD clinic attendees in Manchester, UK, HSV-2 seroprevalence was 14% (10% in men, 19% in women) (Woolley *et al.*, 2000). Among genitourinary medicine clinic and antenatal clinic attendees in Middlesbrough, the prevalence of HSV-2 antibodies is 22% and 8% respectively (Opaneye & Bashford, 2002). These data suggest that the overall prevalence for HSV-2 infection is lower in the UK than in the USA, and that both countries show similar trends in age and gender associated risk for HSV-2 infection.

1.4.2.1 HSV-1 as a cause of genital herpes

Recently, the number of genital herpes cases caused by HSV-1 has been increasing. For example in the USA, of 1145 STD clinic attendees who had a positive diagnosis of genital herpes, 17% had HSV-1 isolated from genital ulcers, with greater

prevalence observed among men who have sex with men (47%) than women (21%) or those of heterosexual orientation (15%). In addition, genital HSV-1 infection was four times more likely to occur in individuals of white ethnicity than non-white ethnicity (Lafferty *et al.,* 2000). Several European studies have explored the changing prevalence of HSV-1 and HSV-2 as a causative agent of genital herpes. Recently, HSV-1 has accounted for more than 50% of first-episode cases in England (Vyse *et al.,* 2000). During the last decade, studies conducted in England (Sheffield, Newcastle upon Tyne, and Watford) and Scotland (Edinburgh, Fife and Glasgow) reported that HSV-1 accounted for 22-71% of cases of genital herpes with raising rates evident in young female patients with first-episode disease (Woolley & Kudesia, 1990; Ross *et al.,* 1993; Tayal & Pattman, 1994; Slomka *et al.,* 1998; Thompson, 2000; Scoular *et al.,* 2002). A study of GUM attendees in Edinburgh found that genital herpes due to HSV-1 was more common in patients born in the UK (Ross *et al.,* 1993). The reason for the increasing proportion of HSV-1 genital infections is speculative. Likely explanations include reduced HSV-1 acquisition in childhood and an increase in oral sex practices the last two decades which would lead to oral and genital HSV-1 infection in adults.

1.4.3 Developing world

Globally, genital herpes is the main cause of STD-related genital ulceration followed by syphilis and chancroid (Corey, 2000). In support of this observation, a study of 560 patients with genital ulcer disease (GUD) in South Africa identified HSV-2 in 36% of all lesions, which made HSV-2 the most common cause of GUD (Chen *et al.,* 2000). In sub Saharan Africa, HSV-2 prevalence is high with HSV-2 seropositivity rates

between 40-50% among 20 year olds in the general population despite HSV-1 acquisition (Kamya *et al.,* 1995; Greenblatt *et al.,* 1988; Gopal *et al.,* 2000). This might reflect the dynamics of sexual mixing between geographical regions and populations, age at first sexual intercourse, safe sexual practices, access to medical care and presence of other sexually transmitted infections. In addition, some serological assays may have varying specificities in different geographical populations which might explain geographical disparity in HSV seroprevalence rates (Hogrefe *et al.,* 2002).

1.4.3.1 HSV-2 prevalence in the general population

In rural community studies in Mwanza, Tanzania 44% of women and 24% of men are HSV-2 seropositive. Seroprevalence was seen to increase with age at about 30 years, and reach a plateau of 75% in women and 50% in men (del Mar Pujades Rodriguez *et al.,* 2002) (Table 1.4). In rural Ugandan adults aged between 25-29 years, HSV-2 seroprevalences of 74% and 45% have been observed among men and women respectively (Kamali *et al.,* 2003). Cross-sectional surveys of four urban African populations in three cities found over 50% of women and over 25% of men to be HSV-2 seropositive in Yaoundé, Cameroon; Kisumu, Kenya; and Ndola, Zambia (Weiss *et al.,* 2001). HSV-2 seroprevalence was seen to increase with age and was the highest among 15-29 year olds. In Addis Ababa, Ethiopia, 61% of women and 50% men were documented to be HSV-2 seropositive with predictors of HSV-2 infection being older age, increasing number of lifetime sexual partners and positive HIV-1 serology (Mihret *et al.,* 2002).

Table 1.4. HSV-2 seroprevalence in the general population in the developing world

Country	Cohort	Sex	Age* (range)	n	% HSV-2+	Ref
Africa						
Uganda 1990-1993	Random sample of residents from 15 neighbouring villages	F/M	(15-≥45)	541/367	71/36	Kamali *et al.*, 1999
Gambia 1999	Random sample of rural women	F	(15-54)	1317	32	Walraven *et al.*, 2001
Ethiopia 1996	General population		(15-≥45)	506	35	Mihret *et al.*, 2002
Tanzania 1991-1994	Sample of 12 rural communities in the Mwanza region		(15-54)	1092	27	Del Mar Pujades Rodriguez *et al.*, 2002
Central and South Asia						
Costa Rica 1984-1985	National, random sample	F/M	(25-59)	766	39	Oberle *et al.*, 1989
Peru 2000-2002	Sample of 34 neighbourhoods		(18-30)	1635	25	Konda *et al.*, 2005
Asia						
China 1995	Random population	F/M	≥25	76/75	18/17	Lo *et al.*, 1999

*Age expressed as mean/median in years unless otherwise stated; F, female; M, male

1.4.3.2 HSV-2 prevalence among high-risk populations

HSV-2 prevalence rates vary among high-risk populations (Table 1.5). A multi-centre study involving sex workers in four different African cities reported HSV-2 prevalence rates of 91% in Cotonou, 84% in Yaoundé, 94% in Kisumu and 87% in Ndola (Morison *et al.,* 2001). In Lagos, Nigeria the seroprevalence of HSV-2 among commercial sex workers (CSWs) is 59% (Dada *et al.,* 1998). HSV-2 seroprevalence of 43% has been reported among STD clinic attendees in Dar-es-Salaam, Tanzania, with 63% of women and 36% of men being HSV-2 antibody positive (Langeland *et al.,* 1998).

The epidemiology of HSV-2 among populations in Central and South America differs from those of sub Saharan Africa. Among female sex workers (FSWs), 61% from Mexico City (Conde-Glez *et al.,* 1999) and 86% from the Mexican-Guatemalan border (Uribe-Salas *et al.,* 2003) are reported to be HSV-2 antibody positive. The prevalence of HSV-2 infection is documented to be much lower in Asia and the Indian sub-continent (ISC) than those in Africa or Central and South America (Weiss, 2004). A study of HIV-1 negative patients in Pune, India reported 38% of male and 51% of female STD clinic attendees to have HSV-2 antibodies compared to 89% in FSWs (Reynolds *et al.,* 2003). However, the study highlighted a number of potential limitations. Differential follow-up rates between high-risk individuals (those with commercial sex worker contacts and greater lifetime numbers of sex partners) and low-risk individuals could have resulted in bias.

Table 1.5. HSV-2 seroprevalence in high-risk populations in developing countries

Country	Cohort	Sex	Age* (range)	n	% HSV-2+	Ref
Africa						
South Africa 1989-1993	STD clinic attendees with GUD	F/M	(15-65)	554	49	Chen *et al.*, 2000
South Africa (NG)	Commercial sex workers	F	(NG)	416	84	Ramjee *et al.*, 2005
Nigeria 1990-1991	Commercial sex workers	F	(12-50)	470	59	Dada *et al.*, 1998
Central African Republic 1998-1999	HIV-1 positive women	F	(15-48)	58	91	Mbopi-Keou *et al.*, 2000
Malawi 1998	HIV-1 positive factory workers	M	(18->45)	279	88	Sutcliffe *et al.*, 2002
Nigeria 1992-1994	Commercial sex workers	F	(15-->45)	796	59	Dada *et al.*, 1998
Tanzania Pre-2002	STD clinic attendees with GUD	F/M	29	70	79	Mwansasu *et al.*, 2002
Tanzania 1989-1993	STD clinic attendees	F/M	27	294	43	Langeland *et al.*, 1998
Cotonou Yaoundé Kisumu Ndola 1997-1998	Commercial sex workers	F	(24-32) (22-34) (21-30) (20-28)	433 328 300 332	91 84 94 87	Morison *et al.*, 2001
Central and South Asia						
Mexico City 1992	Commercial sex workers	F	(16->37)	997	61	Conde-Glez *et al.*, 1999
Mexico 1998	Commercial sex workers	F	(15-47)	460	86	Uribe-Salas *et al.*, 2003
Chile 2003	STD clinic attendees	F/M	(14-70)	200	43	Martinez *et al.*, 2005
Asia						
India 1993-2000	STD clinic attendees	F/M	<20->30	2732	43	Reynolds *et al.*, 2003
Bangladesh 1998	Commercial sex workers	F	>50 (18-30)	203	63	Rahman *et al.*, 2000

*Age expressed as mean/median in years unless otherwise stated; (NG) data not available; F, female; M, male

1.4.4 Risk factors for genital herpes

Several epidemiological studies have estimated the risk of genital herpes acquisition to correlate with increasing number of sexual partners, older age, younger age of sexual intercourse, unprotected intercourse, past history of STDs, ethnicity, gender and socioeconomic status (Nahmias *et al.,* 1990; Fleming *et al.,* 1997; Obasi *et al.,* 1999; Wald *et al.,* 1997). A seroepidemiological survey of 40,000 sera from different populations on all five continents revealed an increase in risk of acquisition with increasing number of lifetime sexual partners (Nahmias *et al.,* 1990). In a study in Milan, the prevalence of HSV-2 was 50% in individuals who had more than five sexual partners in the previous year, compared to 17% in those who had less than two sexual partners (Cusini *et al.,* 2000). Similar findings have been reported in a study from Costa Rica, where 28% of monogamous women were seropositive compared to 75% among those with four or more partners (Rodriguez *et al.,* 2003). The number of years of sexual activity can also increase HSV-2 acquisition (Breinig *et al.,* 1990).

Numerous studies have shown HSV-2 infection to occur more frequently inwomen than men (Gottlieb *et al.,* 2004; Fife *et al.,* 2004; Bunzli *et al.,* 2004). This reflects more efficient male to female transmission and sexual contact with older partners who are more likely to be positive. However, the latter study had a 53% participation rate challenging the representation of the data. In addition, the limited age range (25-74 years) in the study does not provide information about the HSV prevalence rates among adolescents (Bunzli *et al.,* 2004). One prospective study of HSV-2 discordant monogamous couples found that the yearly rate of female to male transmission was 4.5%, whereas that from male to female was 19% (Mertz *et al.,* 1992). Ethnic origin

has shown to influence HSV-2 seropositivity in many large-scale studies. The population-based AIDS in Multiethnic Neighbourhoods (AMEN) study, found highest HSV-2 seropositive rates in African-American women than Caucasian women and men (Siegel *et al.,* 1992). In the USA, HSV-2 seropositivity was higher among black ethnicity (Armstrong *et al.,* 2001; Fleming *et al.,* 1997; Turner *et al.,* 2003). However, these results must be interpreted with caution as with any statistical model, the estimates and confidence intervals are conditional on the assumptions of the model (Armstrong et al., 2001). Increasing age is also associated with increased HSV-2 seroprevalence. The seroprevalence of HSV-2 is nearly non-existent in individuals younger than 12 years of age with peaks occurring by 40 years. This identifies the age of acquisition as 15-40 years as expected for a STD (Johnson *et al.*, 1989; Smith & Robinson, 2002). In contrast, HSV-1 seroprevalence rises in a linear fashion to 70 years where approximately 20% of children aged 5 years and younger are HSV-1 positive (Johnson *et al.* 1989). A recent study among women aged between 20-49 years in Norway has shown HSV-2 prevalence to peak at 35 years (Kjetland *et al.,* 2005). Similar findings have been published elsewhere (Evans *et al.,* 2003; Howard *et al.,* 2003). The age-specific increase in HSV-2 prevalence probably reflects exposure to different sexual partners in early adulthood.

1.4.5 Protective effect of condoms

Inconsistent condom use has been reported among 300 male and female GUM attendees in the UK (Handy, 2004). Consistent with these findings, studies in Africa have shown occasional condom use in 14% of men and 17% of women (Maharaj & Cleland, 2004). A survey of sexual behaviour change among high risk groups in

Uganda, have shown little or no change in sexual lifestyle despite the HIV epidemic. Multiple sexual partners, minimal condom use in marital relationships, and unfaithfulness of spouses were incorporated in their lifestyle (Ntozi *et al.,* 2003).

1.4.6 HSV-2 interaction with HIV-1

Evidence suggests that HSV is a risk factor for the transmission of HIV and that HSV reactivation appears to regulate HIV replication. Possible mechanisms include the mucosal disruption leading to increased susceptibility to HIV-1 and influx of CD4 cells to herpetic ulcerations providing increased numbers of target cells for HIV-1 attachment and entry in the genital tract during HSV-2 reactivation (Mbopi-Keou *et al.,* 2005). Several HSV proteins may also increase the expression of HIV-1 (Golden *et al.,* 1992). Current studies have suggested HSV-2 to be the commonest opportunistic infection in HIV-1 positive individuals with prevalence in developed countries reaching up to 90% in MSM and 40-60% in intravenous drug users (Schacker, 2001). As HSV is the most prevalent cause of GUD worldwide (Lafferty, 2002) it is likely that this transmission synergy is an important factor in the spread of HIV-1. In terms of public health importance, understanding the potential role of STDs in the transmission of HIV-1, along with the identification of modifiable co-factors for HIV-1 transmission would have important implications for HIV control.

1.4.6.1 The effect of HSV-2 on HIV-1

Recent studies have highlighted the role of GUD including genital herpes as a risk factor for HIV-1 infection (Greenblatt *et al.,* 1988; Keet *et al.,* 1990; Ahmed *et al.,*

2003; Chen *et al.,* 2000). A two to four fold increased risk of HIV-1 acquisition has been reported in individuals with genital ulcer disease (Wald & Link, 2002). A meta-analysis of 9 cohort and nested case-control studies showed that the population-attributable risk percentages for HIV-1 infection were 19% and 47% with a HSV-2 seroprevalence of 22% and 80% respectively (Wald & Link, 2002).

Several reports have documented a strong association between genital herpes and HIV-1 in several African countries suggesting an increase in genital herpes to be HIV driven (Reynolds & Quinn, 2005). A cross-sectional study conducted among 393 urban women attending healthcare clinics in Zimbabwe, identified a two-fold increased risk in HIV-1 acquisition in HSV-2 seropositive individuals compared to HSV-2 seronegative persons (Mbizvo *et al.,* 2002). In a Tanzanian study among HIV-1 seropositive GUD patients, 71% from Dar es Salaam and 46% from Mbeya were co-infected with HSV-2 (Ahmed *et al.,* 2003). With recent population-based studies from Africa suggesting 90% of individuals with HIV to be co-infected with HSV-2 (Obasi *et al.*, 1999; Gwanzura *et al.*, 1998), an understanding of the mechanisms underlying HIV-1 and HSV-2 pathogenesis is essential.

Studies elsewhere have reported HSV-2 seroprevalence in HIV-1 infected individuals to range from 75% in pregnant women Seattle, USA (Hitti *et al.,* 1997) 64% in women and 54% in men in Frankfurt, Germany (Rabeneau *et al.,* 2002) 65% among STD clinic attendees in Korea (Kim *et al,* 2003), to 91% in women in Bangui, Central African Republic (Mbopi-Keou *et al.,* 2000). In Melbourne, Australia 61% of HIV-1 infected MSM are seropositive for HSV-2 compared to 20% in HIV-1 negative persons (Russell *et al.*, 2001). However discrepancies in data could have arisen as the

HIV positive population were older and had a greater number of sexual partners compared to HIV negative counterparts. Furthermore the study reported a sizeable amount of missing data (12%) specific to the sexual behaviour which could account for statistical differences. Among HIV-1 positive heterosexual men in Baltimore, USA 63% had a positive HSV-2 antibody status compared to 47% HIV-1 negative men (Hook *et al.*, 1992). When compared to women, 78% of HIV-1 infected women had a HSV-2 infection compared to 58% in HIV-1 uninfected persons (Hook *et al.*, 1992). These finding demonstrate a high level of co-infection with HSV-2 and HIV-1 and highlight the importance of HSV-2 in the risk of HIV-1 acquisition and transmission.

Genital lesions have been identified as a major risk factor for HIV-1 transmission in a series of studies conducted in Africa (Kreiss *et al.*, 1989; Cameron *et al.*, 1991). The frequency of HSV-2 clinical recurrence and asymptomatic shedding is much higher in HIV-1 infected individuals than un-infected individuals (Posavad *et al.*, 2004) with evidence of HIV-1 shed at high levels in most genital lesions (Kreiss *et al.*, 1989; Cameron *et al.*, 1991). These data indicate complex interactions to exist between the two viruses and suggest genital herpes to play an important role in the dynamics of HIV-1 infection.

1.4.6.2 The effect of HIV-1 on HSV-2

HIV-1 acquisition is increased in heterosexual men (RR, 2.2; 95% CI, 1.3-3.8) and MSM (RR, 2.1; 95% CI, 1.3-3.4) who HSV-2 seropositive. Several studies in Zimbabwe and Uganda have demonstrated a four to five-fold increased risk of HSV-2

acquisition in persons who are HIV-1 positive (McFarland *et al.,* 1999; Kamali *et al.,* 1999). However information acquired from these studies was limited due to population size. In addition the timing of seroconversion for both infections were not demonstrated and precise measures of sexual behaviour, sexual exposures, period of active genital lesions, viral replication, viral shedding, and physical examinations were lacking. In a recent prospective study assessing the risk of HIV-1 on STD acquisition in Mombasa, Kenya, HIV-1 infection was highly associated with a significantly higher incidence of GUD (McClelland *et al.,* 2005).

The frequency and severity of HSV-2 shedding has shown to be higher in HIV-1 infected individuals (Augenbraun *et al.,* 1995). Asymptomatic and symptomatic HSV-2 shedding is four times more frequent in HIV-1 infected women than in control groups, with higher levels shed in women with low CD4 counts (Augenbraun *et al.,* 1995). Rates of HSV-2 shedding in the oral cavity and genital tract of HIV-1 infected women were 70% and 79% respectively (Augenbraun *et al.,* 1995). The above findings need to be interpreted with caution as HSV-2 shedding measured at a single time point may not necessarily indicate overall shedding rates. It therefore remains plausible that a greater proportion of patients may have shed the virus on days preceding or following clinical evaluation. Among pregnant HIV-1 positive women HSV-2 shedding at delivery may be four times more common in (Hitti *et al.,* 1997).

Among HIV-1 infected MSM, HSV-2 shedding may be six times more frequent than uninfected individuals (Schacker *et al.,* 1998a). HSV-2 shedding has shown to occur in 10% of 4167 days for HIV-1 positive men compared to 3% of 766 days for HIV-1 negative men (Schacker *et al.,* 1998a). In addition, symptomatic recurrences and HSV

acyclovir-resistant virus strains were also more prevalent in HIV-infected men (Severson & Tyring, 1999).

HIV-1 has also been isolated from genital ulcers (Cameron *et al.*, 1989). In a study of HIV-1 infected HSV-2 seropositive men, HIV-1 RNA was isolated from 25/26 genital herpes episodes and on 66% of days (Schacker *et al.*, 1998b) which suggest that herpetic lesions may facilitate the spread of HIV-1.

1.4.6.3 Biological mechanisms underlying the HSV-2 and HIV-1 synergy

Many theories have been put forward to elucidate the mechanism for increased HIV-1 infection during HSV-2 reactivation. Genital ulcers may provide a portal of entry for HIV-1, while ulcers in HIV-1 infected individuals may shed HIV-1 in ulcer exudate (Levin, 1993). The deposition of HIV-1 infected genital fluids on to the HSV-2 infected epithelial site may be enough to initiate HIV-1 infection. In addition, the disruption of the epithelial barrier by HSV-2 reactivation and infiltration of activated CD4 positive cells and macrophages to the herpetic lesion may create an ideal microenvironment for HIV-1 replication.

One study reported co-infection of human CD4 positive cells with HSV-2 and HIV-1 resulted in unidirectional accelerated replication of HIV-1 (Kucera *et al.*, 1990). The degree of immunosuppression influenced by HIV-1 infection is thought to influence HSV-2 reactivation. There is a direct relation between CD4 positive cell count and the rate of HSV-2 reactivation. In a study of 68 HIV-1 positive men, the OR of anogenital HSV-2 shedding among those with a CD4 positive count of <200 cells/mm^3 was 2.5

(95% CI, 1.1-5.4) when compared to a positive CD4 count of >500 cells/mm^3 (Corey *et al.,* 1983; Schacker *et al.,* 1998a). In addition, HIV-1 infection and the resultant loss of immune function are associated with an increased severity and duration of HSV lesions, as well as increased frequency and duration of HSV-2 reactivation (Schacker *et al.,* 1998a). There also is supporting evidence from studies that show HSV-2 and HIV-1 also interact at a molecular level, and up-regulate HIV-1 replication through the long-terminal repeat (LTR) region (Golden *et al.,* 1992).

1.5 HOST RESPONSES TO HSV-2

1.5.1 Immune mechanisms

T cells are classified into CD4 and CD8 cells with an additional subdivision of CD4 helper cells into T helper (Th) I and II types. CD4 cells may be further classified into those with a Th1 pattern of cytokine secretion and those with a Th2 pattern. The Th2 pattern is predominantly characterised by the secretion of cytokines including IL-4, IL-5 and IL-13 and the production of antibodies. The Th1 pattern leads to the production of cytokines such as IFN- γ, IL-12 and TNF-α, and leads to the activation of CD8 T-cells through IL-12 and IFN- γ.

Development of specific T-cell and B-cell immune responses against HSV antigens takes several days. Innate mechanisms of immunity include interferons (IFN), macrophages and natural killer cells which form the first line of defence (Cunningham & Mikloska, 2001). In addition, neutralising antibodies and specific T lymphocytes with cytotoxic activity secrete antiviral cytokines. Immune mechanisms in recurrent

HSV-2 genital infection are activated quickly, leading to shorter duration of lesions and viral shedding. In recurrent infections, the median duration of lesions and viral shedding are 10 days and 4 days respectively, compared to 21 days and 10 days in primary infections (Corey, 2002).

Several studies have highlighted the role of T-cell responses in HSV disease (Koelle & Corey, 2003) (Figure 1.5a, Figure 1.5b). Immune evasion by HSV is thought to occur by the down-regulation of MHC class I responses, via an inter-play with HSV infected cell protein ICP47 and transporter associated with antigen presentation (TAP) molecule (Figure 1.6). This results in the infected cell escaping the scrutiny of cytotoxic CD8 lymphocytes (Koelle & Corey, 2003). Studies on biopsies of human recurrent HSV lesions have shown that CD4 cells and macrophages infiltrate around the infected cell (Cunningham *et al.,* 1985). CD4 cells producing interferon-γ (IFN- γ) has been detected in vesicle fluid as demonstrated in several studies (Cunningham *et al.,* 1985; Cunningham & Merigan, 1984; Torseth & Merigan, 1986). IFN- γ can restore MHC class I expression on the epidermal surface and increase the cellular production of TAP leading to MHC class II expression. Cells expressing MHC class II can be recognised by specific activated CD4 resulting in the increased production of IFN- γ mediated by CD8 cells (Cunnigham & Noble, 1989) (Figure 1.6). MHC class I expression allows recognition by CD8 cells which destroy virus infected cells (Mikloska *et al.,* 1996).

Cytokine production within herpetic lesions are critical for controlling HSV-2. Similarly, the role of CD8 cells in controlling HSV-1 infection in the trigeminal ganglia has been reported (Decman *et al.,* 2005; Liu *et al.,* 2001; Khanna *et al.,* 2004).

Elevated levels of IFN-γ and TNF-α transcripts in latently infected trigeminal ganglia has shown TNF-α to synergize with IFN-γ in controlling HSV-1 replication *in vitro* and *in vivo* (Theil *et al.,* 2003; Feduchi *et al.,* 1989; Paludan *et al.,* 2001). It is thought that HSV glycoprotein gD induces TNF-α secretion in macrophages and that post-entry events are required for full induction (Paludan *et al.,* 2001).

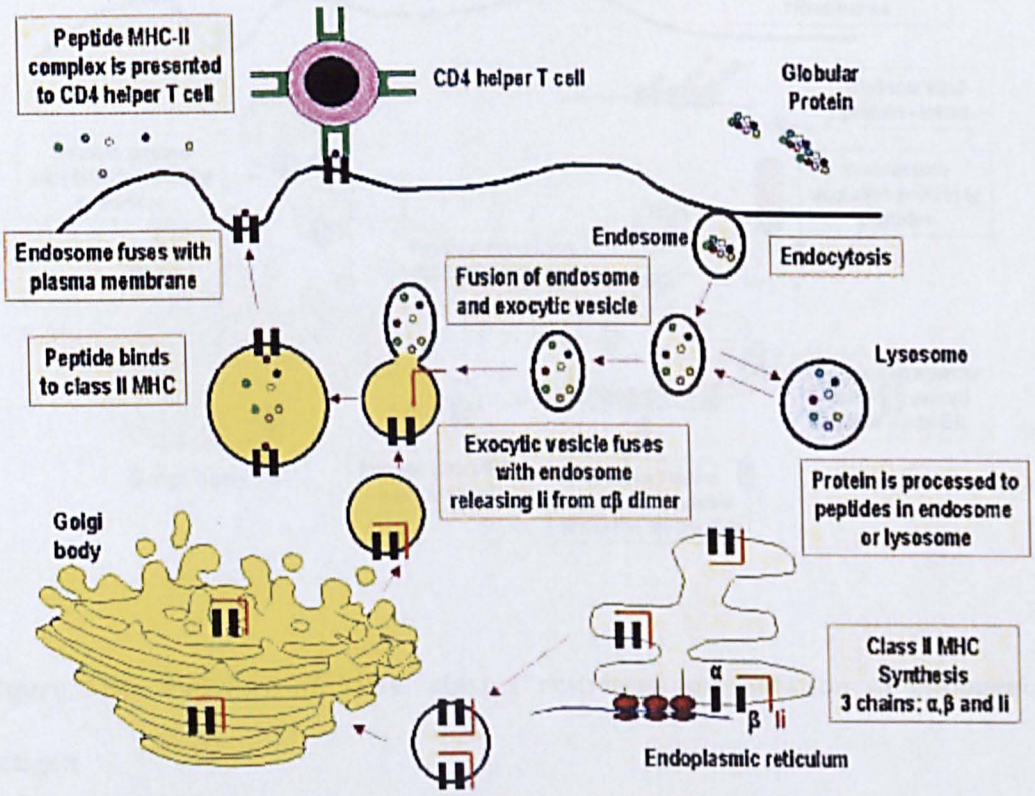


Figure 1.5a Pathway of MHC class II restricted presentation of exogenous antigen

Antigen presenting cells (APC) comprising of macrophages, dendritic cells (Langerhans cells), and B cells express MHC class II whose expression may be induced by interferon-γ in the case of macrophages. Steps involved in the MHC class II pathway are: Endocytosis and fragmentation of exogenous proteins in an endosome. Synthesis and assembly of MHC class II chains in the endoplasmic reticulum,

followed by transportation through the Golgi apparatus to the endosome for further fragmentation. Association of exogenous peptide fragments with class II MHC molecules and their transportation to the cell surface.

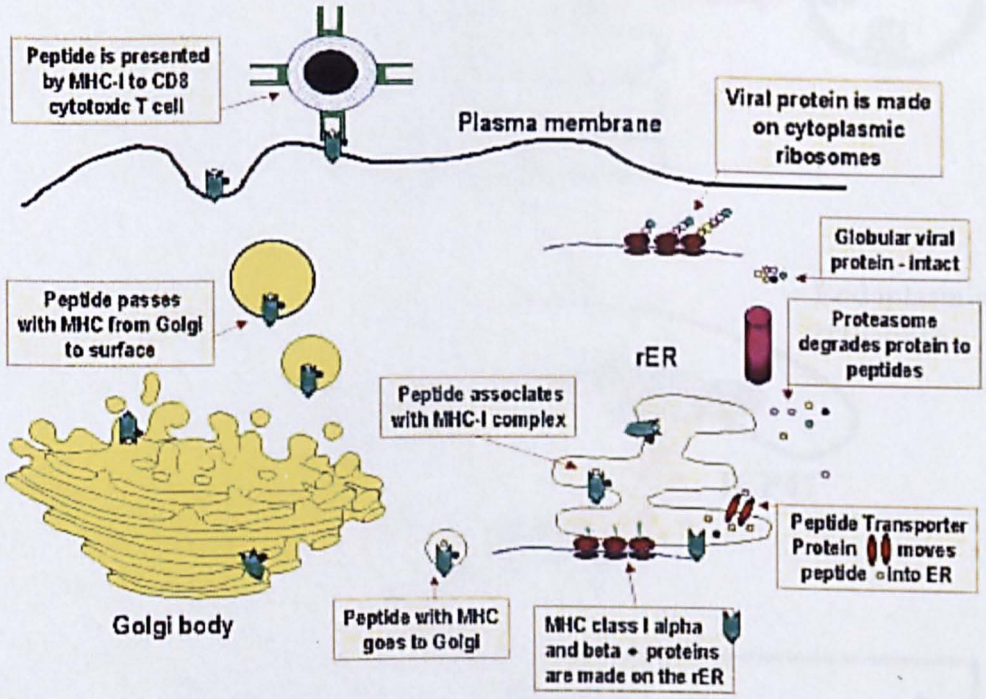


Figure 1.5b Pathway of MHC class I restricted presentation of endogenous antigen

All nucleated cells express class I MHC. The stages for MHC class I processing include: Fragmentation of proteins in the cytosol by proteosomes or by other proteases, transportation of fragments across the endoplasmic reticulum membrane by transporter proteins, synthesis and assembly of class I heavy chain and β_2 microglobulin in the endoplasmic reticulum followed by transportation of this stable complex to the cell surface.

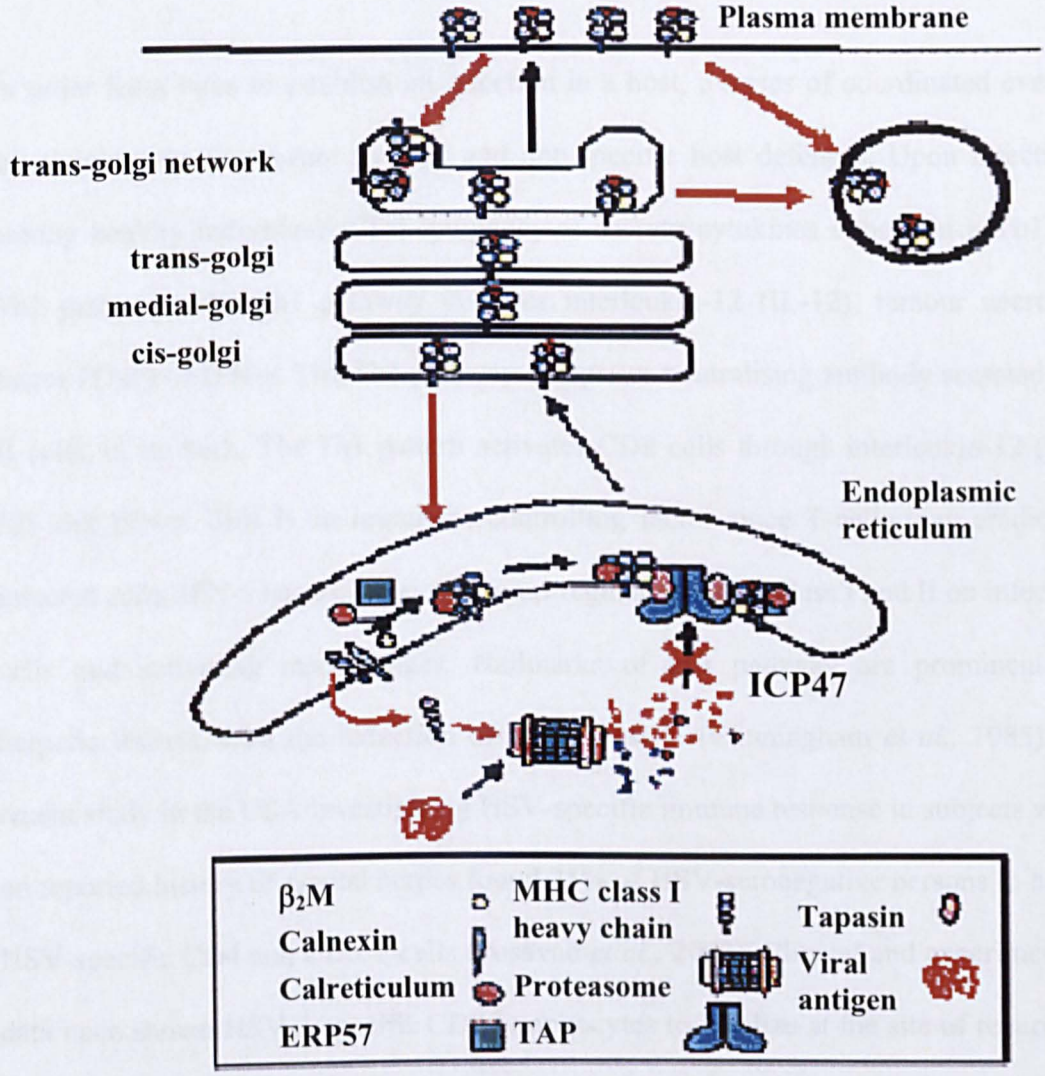


Figure 1.6 HSV inhibition of the MHC class I antigen presentation pathway.

TAP is a heterodimer whose membrane domains form the peptide binding region and a pore through which proteins are translocated. TAP is a target for viral inhibition as peptides presented by MHC class I molecules are generated in the cytosol and require translocation across the endoplasmic reticulum membrane. HSV ICP47 acts as a competitive inhibitor and prevents peptide translocation by TAP.

1.5.2 Immune pathways and HIV-1

In order for a virus to establish an infection in a host, a series of coordinated events are required to circumvent specific and non-specific host defences. Upon infection among healthy individuals CD4 lymphocytes secrete cytokines either via a Th1 or Th2 pathway. The Th1 pathway involves interleukin-12 (IL-12), tumour necrosis factor (TNF) or IFN-γ. The Th2 pathway comprises neutralising antibody secreted by B cells, or by both. The Th1 pattern activates CD8 cells through interleukin-12 (IL-12) and IFN-γ. This is an important controlling factor since T-cells may eradicate infected cells. IFN-γ has a critical role in up-regulating MHC class I and II on infected cells and activating macrophages. Hallmarks of this pathway are prominent in herpetic lesions, with the induction of MHC class II (Cunningham *et al.,* 1985). A recent study in the USA investigating HSV-specific immune response in subjects with no reported history of genital herpes found 25% of HSV-seronegative persons to have HSV-specific CD4 and CD8 T cells (Posavad *et al.,* 2003). Clinical and experimental data have shown HSV-2-specific CD8 lymphocytes to localize at the site of recurrent HSV-2 genital lesions (Koelle *et al.,* 1993; Koelle *et al.,* 1994). The clearance of infectious virus from lesions has shown to be temporally correlated with the infiltration of CD8 T cells and HSV-specific lymphocytes (Koelle *et al.,* 1998). Similar observations on the inhibition of HSV-1 replication in latently infected cells of the trigeminal ganglia in murine models have also been documented (Decman *et al.,* 2005; Liu *et al.,* 2000).

HIV-1 curtails the immune system, by destroying CD4 infected and uninfected bystander cells. In the first few weeks after HIV-1 infection, the numbers of CD4 cells

decline while those of CD8 rise sharply (Pattanapanyasat & Thakar, 2005). The half-life of most HIV-1 infected T cells *in vivo* is 12-36 hours (Ho *et al.,* 1995). The decline in immune function governed by the loss of CD4 cells is thought to make the host susceptible for opportunistic infections, although the specific mechanisms underlying the loss of CD4 cells and the varied susceptibility of HIV-1 infected individuals to HSV-2 infections still remains unclear (Pattanapanyasat & Thakar, 2005). Apoptosis is thought to contribute towards T-cell destruction, as elevated levels of apoptosis have been found to correlate directly with disease progression and inversely with Th cell counts (Gougeon *et al.,* 1996). HIV proteins gp120, Tat, Nef and Vpu have been shown to induce cell death in uninfected CD4 cells (Paranjape, 2005). Syncytium formation of infected and uninfected cells stimulated by gp120 can also contribute to cell death (Alimonti *et al.,* 2003). In addition, continuous budding of viruses from infected cells may cause membrane disruption and increased permeability leading to the death of the cell.

HIV-1 infection and subsequent immunosuppression in individuals provide a unique model system to study critical immune functions necessary for control and resolution of human HSV infections. One hypothesis is that quantitative differences in specific T cell responses to HSV-2 may determine the development of genital herpes (Posavad *et al.,* 1997). In a cross-sectional study of individuals co-infected with HIV-1 and HSV-2 in Washington, USA, the frequency of HSV-specific CD8 cells was correlated inversely with the severity of recurrent anogenital HSV-2 infection (Posavad *et al.,* 1997). Another study documented T cell activity to be low among cells derived from early culture-positive lesions, than those at later stages. In addition, viral clearance from the lesion was associated with a high level of local cytolytic activity (Koelle *et*

al., 1998). The data generated from this study was limited by technical differences. Sampling only a portion of the herpes lesion may have missed T cells that localized to a fine region, and that some cells present in biopsies may have failed to expand within the culture conditions used in the study.

Several studies have demonstrated HIV-1 positive individuals have significantly lower HSV-specific proliferative responses compared to HIV-1 negative HSV seropositive patients (Hersh *et al.,* 1985; Wainberg *et al.,* 1987). This observation has been correlated with absolute $CD4^+$ lymphocyte counts (Hersh *et al.,* 1985; Carrega *et al.,* 1995). A study in Washington, USA showed the frequency of HSV-specific CD8 lymphocyte precursors in HSV-HIV-1 co-infected individuals to be significantly lower than in HIV-1 negative HSV seropositive individuals (P = .0005) (Posavad *et al.,* 1997). In the same study, HIV-1 infected patients who suffered more severe genital herpes recurrences had significantly lower HSV-specific CD8 lymphocyte precursor frequencies than those patients with mild recurrences (P = 0.03).

Data on highly active antiretroviral therapy (HAART) in HIV-1 infected persons has demonstrated suppression of HIV-1 replication, with subsequent increases in memory and naive CD4 lymphocyte responses (Posavad *et al.,* 2004). There is very limited data on the effect of HAART on the host immune responses to HSV-2, and its association with HSV-2 reactivation and shedding. Recent data has shown HAART does not significantly reduce the frequency of mucosal HSV shedding in HIV-1 infected persons however, it does improve clinical HSV disease by reducing the percentage of days HSV lesions were presented (Posavad *et al.,* 2004). A key to

understanding the immune mediators of infection among both these viruses may assist in guiding potential preventive strategies.

1.6 ANTIVIRAL MANAGEMENT OF HSV-2 INFECTIONS

Antiviral therapy has shown to decrease viral shedding and symptoms associated with HSV infection. Nucleoside analogues such as acyclovir are mono-phosphorylated by viral thymidine kinase (TK) and subsequently converted to di and triphosphate forms by host enzymes. Acyclovir triphosphate can then compete with the substrate for HSV-encoded DNA polymerase causing termination of HSV DNA replication. Acyclovir has been shown to reduce HSV-2 asymptomatic shedding. In a double-blind placebo-controlled trial of women with a history of genital herpes for <2 years, acyclovir at 400mg administered twice daily for 70 days was shown to reduce HSV-2 sub-clinical shedding (Wald *et al.,* 1996). Similarly, valacyclovir has shown to reduce sub-clinical shedding from 15.3% of days to 0.7% days in men and women (Wald *et al.,* 1996). Famicyclovir has shown to suppress asymptomatic shedding in women with a history of genital herpes (Sacks, 2004).

The transmission of HIV-1 and HSV-2 may be possibly reduced by antiviral agents which decrease virus shedding and virus load (Celum, 2004). A meta-analysis of 8 controlled trials showed acyclovir offered substantial survival benefit in HIV-1 infected patients (Ioannidis *et al.,* 1998). It has been suggested that a reduction in HSV infection may lead to suppression of HIV-1 replication (Severson & Tyring, 1999). The suppression of HSV by acyclovir may also reduce HIV-1 RNA levels (Schacker *et al.,* 1998a). Data assessing the impact of highly active anti-retroviral

therapy (HAART) on HSV-2 shedding is limited. One recent study demonstrated that HIV-1 infected individuals on HAART to have significantly fewer days with HSV lesions compared to un-treated individuals (Posavad *et al.*, 2004). The possible use of anti-viral treatment in conjunction with improved STI management provides a viable strategy in controlling the HIV-1 and genital herpes epidemic.

2. Chapter 2. Materials and Methods

2.1. SPECIMENS

2.1.1 Genital swabs

Genital swabs were collected from GUM patients with suspected genital herpes attending Kings College Hospital, London and placed in 2.5 ml of virus transport medium (VTM) containing minimal essential medium (MEM). MEM constitutes Earle's salts and 25 mM Hepes buffer containing 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) (BioWhittaker Europe, Berkshire, UK) and 10% (v/v) heat inactivated foetal bovine serum (FBS) (Sigma Aldrich, Dorset, UK). The VTM was balanced with 25 mM Hepes (BioWhittaker Europe, Berkshire, UK) supplemented with 10% bovine serum albumin (BSA), 7.5% sodium bicarbonate, penicillin (500 U/ml), streptomycin (1 mg/ml) and amphotericin B (5 µg/ml). After collection, the samples were vortexed for 30 seconds and aliquots of 500 µl were either used immediately for virus isolation or stored at -80°C for PCR and sequencing.

2.1.2 Serum samples

Blood specimens (7.5 ml) were collected in serum collection tubes (Sarstedt, Leicester, UK) from HIV-1 infected and uninfected individuals attending the Royal Free Hospital, London. Whole blood samples were centrifuged at 1500 × g for 15 min, serum isolated and stored at -20°C.

2.1.3 PBMC

Blood samples (15 ml) were collected in preservative-free sodium heparin tubes (Sarstedt, Leicester, UK) for the isolation of PBMC from HIV-1 negative volunteers and HIV-1 infected individuals at different stages of HIV-1 disease from the Royal Free Hospital, London: long-term non progressors, recently diagnosed HIV-1 patients prior and following HAART and HIV-1 patients already on HAART. Isolation of PBMC was performed as described (Section 2.7.1).

2.2 VIRUS ISOLATION IN CELL CULTURE

African green monkey kidney (VERO) cells were used for HSV isolation in cell culture. Vero cells were grown in MEM growth medium in a humidified atmosphere of 5% CO_2 at 37°C and were passaged twice weekly prior to reaching confluency. Passaging was performed by rinsing the cell monolayers with sterile phosphate buffered saline (PBS) followed by trypsinisation for 5 min with 5 ml of Trypsin (0.05%)/EDTA (0.01%) solution (Gibco BRL, Paisley, UK). Upon detachment of cells from the flask surface, the cells were re-suspended in MEM/10% FBS at a ratio of 1:2 or 1:3.

Cells were inoculated with 200 µl of specimen and monitored daily for the development of cytopathic effect (CPE). Cultures were maintained for 10 days. CPE was characterised by the presence of syncytia and formation of giant cells visualised through a light microscope.

2.2.1 Direct Immunofluorescence Test

HSV-1 and HSV-2 infected cell cultures were examined for CPE. Upon observation of a CPE, the infected Vero cells were washed with PBS, scraped off the culture flask and dried on to a Teflon coated slide and air-dried. The slide was then fixed in cold acetone for 10 min at 4°C, and 15 µl of fluorescein-labelled purified murine monoclonal antibodies against HSV-1 and HSV-2 (Syva Microtak, Palo Alto, USA) were added to each well of the slide. Antibody incubation was carried out in a humidified chamber for 15 min, and excessive reagents were washed off by rinsing with PBS for 10 sec. The slides were air-dried and mounted with buffered glycerol and examined under a fluorescent microscope. Positive staining for HSV was identified by green fluorescent staining in the cells. No blind or direct staining on patient specimens was performed.

2.3 PREPARATION OF VIRAL LYSATES

HSV-1 (MacIntyre strain) and HSV-2 (MS strain) (Microbix Biosystems Inc, Ontario, Canada) strains were grown in Vero cells with MEM/10% FBS. Culture supernatants were clarified by centrifugation at 500 × g for 15 min. The pellet of infected vero cells were mechanically disrupted, rinsed with sterile PBS, and suspended in detergent 1% Triton X-100/1mM phenylmethylsulfonylfluoride (PMSF)/0.1% sodium dodecyl sulphate/50mM Tris (pH 8.0). Concentrations of lysates were determined by a protein assay based on bicinchoninic acid (BCA) (Pierce Biotechnology, Illinois, USA). The protein assay was performed using a series of dilutions of known concentrations of a

common protein such as Bovine serum albumin (BSA). Dilutions of stock BSA (2 mg/ml in 0.9% saline and 0.05% sodium azide) were made at 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml according to manufacturer's instructions. Working Reagent was also prepared by mixing 50 parts of BCA Reagent A (500 ml containing sodium carbonate, sodium bicarbonate, binchoninic acid and sodium tartrate in 0.1M sodium hydroxide) to 1 part of Reagent B (25 ml containing 4% cupric sulphate) according to manufacturer's instructions. BSA standard, HSV-1 and 2 lysates (25 µl) and working reagent (200 µl) were added to individual wells of a microplate. The plate was mixed thoroughly on a shaker for 30 sec, covered, incubated at 37°C for 30 min and cooled at room temperature. The absorbance was measured at 562 nm on a plate reader. A standard curve was plotted using the average blank corrected measurements for each BSA standard versus its concentration in mg/ml, and this was used to determine the concentration of HSV-1 and 2 lysates. Virus lysates were stored at 1 mg/ml at -80°C until further use.

2.4 DETECTION OF HSV ANTIBODIES

2.4.1 Enzyme immunoassay (EIA)

The HSV-1 and HSV-2 antibody status of individuals was determined using a modified HSV type-specific IgG EIA (Focus Technologies, Cypress, USA). The reagents were brought to room temperature prior to use. The pre-coated polystyrene plate containing glycoprotein G antigen, was soaked with 100 µl of 1X wash buffer solution (10X solution containing PBS with 0.01% thimerosal) for 5 min and then decanted. Serum samples, controls (negative sera, low positive sera, high positive sera

and a cut-off calibrator provided by the manufacturer) were diluted 1:101 using sample diluent (surfactant in protein–based PBS with 0.001% gentamicin and 0.01% thimerosal). A blank containing sample diluent alone was used. To appropriate wells, 100 µl of sample diluent and 100 µl of diluted serum samples and controls were added. The plate was covered, incubated at 25°C for 60 min to allow specific antigen-antibody binding and washed three times with 100 µl of 1X wash buffer to remove non-specific reactants. To each well, 100 µl of IgG conjugate (affinity-purified peroxidase-conjugated goat anti-human IgG, with 0.05% thimerosal) was added to react with specific IgG. The plate was covered, incubated at 25°C for 30 min and washed a further 3 times with 1X wash buffer as previously described. To each well, 100 µl of substrate reagent (tetramethylbenzidine and hydrogen peroxide in buffer) was added and the plate incubated at 25°C for 10 min for colour development. The reaction was stopped after by adding 100 µl of 1M sulphuric acid. The absorbance was read at 450 nm by a microwell spectrophotometer and index values calculated by dividing the optical density of the specimen by the optical density of the cut-off calibrator. The cut-off for a positive result was raised from 1.1 (recommended by the manufacturer) to 3.1 to increase specificity. Samples with index values between 0.9 and 3.0 were scored as equivocal.

2.4.2 HSV avidity assay

Frozen sera were thawed and sera were tested using a modified protocol of the HSV-2 type-specific IgG EIA (Focus Technologies, Cypress, USA) as described above (Section 2.4.1). Once all the reagents were brought to room temperature, serum samples were vortexed and 2.5 µl of each serum sample was diluted 1:101 with 250

µl of sample diluent, provided in the HSV-2 EIA kit. Diluted sera (100 µl) were added to duplicate wells of the plate. The plate was incubated as described above, and processed with an additional wash step before the addition of the conjugate. The plate was washed with 1X wash buffer (200 µl) to one set of duplicate wells, and 200 µl of Urea buffer [Tris (50 mM/L), urea (6M/L), sodium azide (0.9g/L); pH 7.4] was added to the other wells. After 5 min, the wells were washed twice with 1X wash buffer, conjugate and substrate added, and the plate read as described above (Section 2.4.1). Each sample had 2 optical density values: one from the untreated specimen and one from the urea buffer treated specimen. Index values for the untreated specimens were obtained by dividing their optical density value by the optical density mean of the cut-off calibrator. To obtain the avidity value, the optical density of the treated specimen was divided by the optical density of the untreated specimen and multiplied by 100. HSV-2 index values were established using a previously published avidity assay based on the HerpeSelect HSV-2 EIA (Morrow *et al.*, 2004). Samples with HSV-2 index values > 3 were considered positive, and those with index values between 0.9 and 3.0 were scored as equivocal.

2.4.3 HSV-2 Inhibition EIA

Prior to the EIA, the sera was diluted 1:50 and 1:200 using HerpeSelect sample diluent (surfactant in protein–based PBS with 0.001% gentamicin and 0.01% thimerosal) and then diluted with an equal volume of either HSV-1 or HSV-2 lysates (1 mg/ml) (Section 2.3). The samples were incubated for 60 min on a shaker at room temperature. The samples were then added to the wells of the HSV-2 EIA, incubated for 60 min at room temperature followed by the addition of conjugate and substrate.

The plate was read at 450 nm and index values calculated as previously described. Percent inhibition due to pre-incubation with HSV-2 lysate was determined by the formula [1 - (index of HSV-2 lysate well/index of HSV-1 well)] X 100. Samples showing inhibition ≥60% with HSV-2 but not with HSV-1 lysates in two independent assays were scored as HSV-2 positive.

2.4.4 Immunoblot

The HerpeSelect Immunoblot (Focus Technologies, Cypress, USA) was used to confirm positive and equivocal results generated by the HSV-1 or HSV-2 EIA. The assay employs the use of individual nitrocellulose membrane strips immobilised with native HSV gG-1 or gG-2. All reagents were brought to room temperature before use. A 1X wash buffer solution was prepared from a 10X concentrate (surfactant in PBS with 0.1% azide). This was used to make the specimen buffer, by adding 100 ml of 1X wash buffer to 4 grams of blotting powder (non-fat dry milk). Each strip was washed in 2 ml of 1X wash buffer for 5 min on a shaker. To each strip, 2000 µl of specimen buffer and 20 µl of specimen or control were added. The strips were incubated at room temperature for 60 min on a shaker to allow antigen-antibody binding. Following decantation, the strips were washed using 2000 µl of 1X wash buffer for 3 min on a shaker. To each well, 2000 µl of IgG conjugate (alkaline phosphatase-conjugated goat anti-human IgG, with 0.1% azide) was added, incubated for 30 min at room temperature on a shaker, decanted and washed thrice with 1X wash buffer as previously described. Band formation was observed upon addition of 2000 µl of substrate reagent (bromo-chloro-indolyl phosphate and nitroblue tetrazolium) to each strip on a shaker for 30 min. Colour development was stopped by

decanting the substrate solution and rinsing the strips five times with de-ionised water. Strips were then dried on filter paper. Reactivity was interpreted as gG-1, gG-2, HSV common antigen and anti-human serum bands (Figure 2.1).

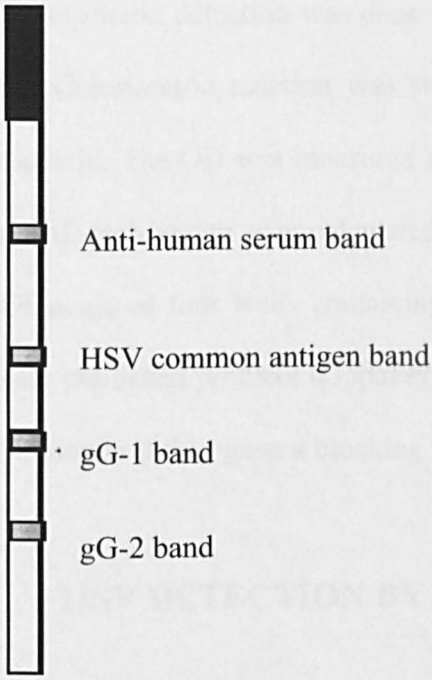


Figure 2.1. Band interpretation of HerpeSelect 1 and 2 immunoblot IgG

2.4.5 gG-2 monoclonal antibody blocking EIA

To further validate our assay, a random subset of 50 sera was sent to the Health Protection Agency (HPA) (Colindale, London, UK) for HSV-2 testing by an in-house gG-2 monoclonal antibody (mAb)-blocking EIA. Briefly, wells of the plate were coated with HSV-2 extracts in PBS (1:25 dilution) for 12 hours at 4°C. Plates were washed with detergent (1.5% Triton X-100 and 0.5% Nonidet P-40 in PBS) for 30 min at 25°C followed by incubation with 10% FCS/PBS for 2 hours at 37°C. The plate was further incubated for 1 h at 37°C with 10% FCS/PBS and 0.2% Tween 20. Dilutions of controls and test sera were as follows: a 1:4 dilution of test sera, a

1:16000 dilution of HSV-2-specific monoclonal antibody, and a 1:1,000 dilution of horseradish peroxidase-conjugated anti-mouse monoclonal antibody. Plates were washed three times between each stage using 0.05% Tween 20 in PBS for 30 min at 25°C. Colorimetric detection was done using TMB substrate at room temperature in the dark. Colorimetric reaction was stopped after 20 min by the addition of 2M sulphuric acid. The OD was measured at 450 nm/620 nm for reference. The percent blocking of each serum was calculated by comparison with controls and with the mean $OD_{450/620}$ of four wells containing a positive control serum as described in a previously published protocol (Gopal *et al.*, 2000). Sera were considered positive for HSV-2 antibody if they gave a blocking value of ≥50%.

2.5 HSV DETECTION BY PCR

2.5.1 Specimen preparation

For the detection of HSV DNA in genital swabs, three different techniques for specimen preparation were employed prior to polymerase chain reaction (PCR).

2.5.1.1 Manual DNA extraction

The QIAamp DNA mini kit (Qiagen, West Sussex, UK) was used to extract HSV DNA from genital specimens, following a modified protocol. To a 200 µl aliquot of genital specimen, 20 µl of Qiagen Protease, and 200 µl lysis Buffer AL were added and vortexed for 15 sec. To improve extraction in samples with low DNA copy numbers, an aqueous solution of 1 µl of poly dA (5 µg/1 µl) (Amersham Biosciences,

Buckinghamshire, UK) was added during lysis. Following incubation at 56°C for 10 min, lysed samples were centrifuged briefly at 150× g for 2 min and 230 µl of 99% ethanol added to the pellet to improve extraction. The lysed specimens were pulse-vortexed for 15 sec, further centrifuged at 150 × g for 2 min and carefully applied to the QIAamp spin column. The collection tubes containing the filtrate were discarded. To the column, 500 µl of buffer AW1 was added, and the column centrifuged at 6000 × g for 1 min. Filtrate in the collection tubes was discarded, and 500µl of buffer AW2 was added. The columns were centrifuged at 20000 × g for 3 min, and the filtrate discarded. DNA from the column was eluted in 60 µl of deionised water (Sigma, Dorset, UK), incubated at room temperature for 5 min, and centrifuged at 6000 × g for 1 min. The DNA filtrate was stored at -80°C.

2.5.1.2 Automated DNA extraction

The MagNA Pure LC (Roche Diagnostics, East Sussex, UK) is a robotic DNA extraction system involving magnetic glass particle technology. Automated pipetting of samples (200 µl) was carried out by the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics, East Sussex, UK). Each run had 4 negative controls and DNA was eluted in 100 µl of deionised water and stored at -80°C.

2.5.1.3 Virus precipitation

Virus precipitation was performed by adding 200 µl of the genital specimen in VTM to a mixture of 2.5M NaCl (25 µl) and 20% PEG 8000 (25 µl). The mixture was vortexed, incubated on ice for 30 min, and centrifuged at 11,000 × g for 20 minutes at

4°C. The supernatant was aspirated, pellet re-centrifuged at 4°C for 5 minutes. The supernatant was aspirated and the pellet was re-suspended in 20 µl of de-ionised water and stored at -80°C.

2.5.2 Real-time PCR

Real-time PCR by LightCycler technology (Roche Diagnostics, East Sussex, UK) was used to type HSV isolates by PCR amplification of the DNA polymerase gene. This method used high temperature transition rates (20°C/sec) in specially designed glass capillary reaction vessels. Real-time monitoring of amplified product was performed using a pair of fluorescently labelled hybridisation probes in close proximity (< 5 nucleotides separation) results in fluorescence resonance energy transfer (FRET) between the fluorophores. The amount of fluorescence measured was proportional to the amount of specific PCR product in the reaction mix. PCR quantification can be performed by determining the crossing point value. The crossing point is the cycle at which PCR amplification begins its exponential phase and is proportional to the logarithm of the initial concentration. As the crossing-point value increases, the starting concentration of sample target sequence decreases. Typing was characterised by melting curve analysis.

2.5.2.1 PCR preparation and amplification

HSV DNA from genital swabs was used as a template for PCR amplification reactions using primers specific for the 215 base pair region of the DNA polymerase gene. The PCR protocol was modified from a previously described method (Burrows

et al., 2002; Espy *et al.*, 2000). Amplification was performed using the Roche LC Fast Start DNA Master Hybridisation Probe kit (Roche Diagnostics, East Sussex, UK). Briefly, 60 µl of LightCycler FastStart Reaction Mix Hybridisation Probes was added to the vial containing LightCycler FastStart enzyme (HotStart reaction mix containing Taq DNA polymerase, reaction buffer, DNTP mix with dUTP instead of dTTP and 10mM magnesium chloride) followed by gentle mixing. To this, magnesium chloride (4 mM), forward (HSV POL F 5'-GCTCGAGTGCGAAAAAACGTTC-3') and reverse primers (HSV POL A 5'-TGCGGTTGATAAACGCGCAGT-3') (5 µM) and two fluorescently labelled hybridisation probes HSV-2 FLU (5'-gCgCACCAgATCCACgCCCTTgATgAgC-FAM-3') and HSV-2 LCR (5'-LC Red 640-CTTgCCCCCgCAgATgACgCC-phosphate-3') (1 µM) were added (Table 2.1). A total volume of 20 µl was obtained by adding 5 µl of DNA or precipitated virus to a PCR reaction mix in glass capillaries.

Table 2.1. Reaction components and volumes for DNA amplification by the LightCycler

Components	Volume (µl)
FastStart DNA Master Hybridisation Probe	2.0
HSV forward primer (5 µM)	2.0
HSV reverse primer (5 µM)	2.0
HSV FLU Probe (1 µM)	2.0
HSV LCR probe (1 µM)	2.0
Magnesium chloride (4mM) supplied in the LC FastStart DNA Master Hybridisation Probe kit	2.4
De-ionised water supplied in the LC FastStart DNA Master Hybridisation Probe kit	2.6
DNA	5
Total volume	20

The RealArt™ HSV1/2 LC PCR kit (Artus Biotech, Hamburg, Germany) was also used to process specimens for LightCycler PCR. Briefly, to 5 µl of specimen DNA, 15 µl of RealArt HSV 1/2 Mastermix was added to LightCycler capillaries at 4°C and centrifuged at 150 × g for 10 sec. In addition, a quantification standard for HSV-1 and HSV-2 were processed along with a negative control with DNA substituted with de-ionised water.

Reactions were performed under the following conditions: one 1 cycle of initial denaturation at 95°C for 10 min (temperature transition rate of 20°C/sec) to activate DNA Taq polymerase, followed by 50 cycles of denaturation at 95°C for 5 sec (20°C/sec transition), annealing at 50°C for 5 sec (20°C/sec transition) with single fluorescence acquisition, and extension at 72°C for 5 sec (20°C/sec transition). Melting curve analysis was performed as follows: annealing at 40°C for 30 sec (20°C/sec transition) followed by an increase to 80°C for 0 sec (0.1°C/sec transition) with continuous fluorescence acquisition, and cooling to 40°C for 30 sec (20°C/sec transition). The Tm of DNA fragments and hybridisation probes was used to differentiate between HSV types. The probe Tm was 59°C for HSV-1 and 64°C and/or 71°C for HSV-2.

2.5.2.2 Cloning and PCR quantification

The HSV-1 and HSV-2 DNA polymerase gene amplified by real time LightCycler PCR was used as a control for quantification. After PCR amplification, electrophoretic separation of PCR products (10 µl) was performed on 2% agarose gels in 0.5 × Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized by

UV illumination. Purification of HSV DNA from the agarose gel was performed with the QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK). Briefly, the DNA band on the gel was excised and weighed. Two volumes of diffusion buffer were added to 1 volume of gel followed by 30 min incubation at 50°C. The sample was centrifuged at 11000 × g for 1 min, supernatant removed and filtered through a column containing Whatman GF/C filter to remove residual polyacrylamide. Three times the volume of buffer QG was added to one volume of the supernatant and mixed. The suspension was filtered through the QIAQuick Spin column, centrifuged at 10000 × g for 1 min, and the filtrate discarded. To the column, 750 µl of Buffer PE was added and the column centrifuged at 10000 × g for 1 min. The filtrate was discarded and the column centrifuged at 20000 × g for 3 min. DNA from the column was eluted in 50 µl of deionised water (Sigma, Dorset, UK), incubated at room temperature for 5 min, and centrifuged at 6000 × g for 1 min.

The amplicon was cloned directly into pGEM®-T easy vector using the pGEM®-T Easy Vector System (Promega, Southampton, UK). Briefly, the pGEM T easy vector and control insert DNA tubes were centrifuged. Ligation reactions were set up using 5 µl 2X rapid ligation buffer, 1 µl pGEM®-T easy vector (50 ng/µl), 1 µl of PCR product, 1 µl T4 DNA Ligase (3 Weiss units/µl) and deionised water to make a total volume of 10 µl. Reactions were mixed and incubated at 25°C for 60 min. Transformation were performed using JM109 competent cells provided in the kit.

Briefly, 50 µl of competent cells were incubated with 5 µl of the ligation reaction on ice for 20 minutes and heat shocked at 42°C for 45 seconds to allow uptake of the ligated insert-vector. Reactions were incubated on ice for 2 min followed by a further

incubation at 37°C for 90 min in 950 µl of 2 x Luria-Bertani (LB) medium (Sigma, Dorset, UK) with gentle agitation in a shaking incubator. LB agar plates containing ampicillin (100 µg/ml) were spread with 100 µl of 100mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase, (X-Gal). Plates were incubated at 37°C to facilitate the screening of blue and white colonies and stored at 4°C. The transformation suspension was centrifuged at 6000 × g for 1 min and 900 µl of the supernatant was removed. The cells were re-suspended in the remaining 100 µl of medium, spread onto the plates and incubated at 37°C for 16-24 hours. Plasmid DNA was extracted from recombinant colonies using a Wizard^{R} *Plus* SV Minipreps DNA Purification System kit (Promega, Southampton, UK) kit. Briefly, single, well-isolated recombinant colonies were selected and grown in 5 mls of LB and incubated at 37°C overnight in a shaking incubator at 250 rpm. Cells (4 ml) were harvested by centrifugation and re-suspended in a Tris-EDTA-RNase A solution (50mM-10mM-100 µg/ml). After alkaline lysis at room temperature, the cells were neutralised (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) and passed through a spin column comprised of a resin matrix to which the DNA selectively binds. Contaminants were removed by applying high salt-ethanol washes to the column (60mM potassium acetate, 8.3mM Tris-HCl, 0.04mM EDTA and 60% ethanol). Purified DNA was eluted from the resin matrix with 100 µl nuclease free water. The identity of individual clones was confirmed by sequencing both strands of the viral DNA insert.

Restriction digests of the recombinant plasmid DNA were carried out in a total volume of 30 µl as described in the manufacturer's protocol (Promega, Southampton,

UK). DNA (10 µl) was incubated for 90 minutes at 37°C with Pst *I* as described recently (Burrows *et al.,* 2002) to linearise plasmid DNA. DNA purification from the agarose gel was performed using the QIAquick Gel Extraction kit (Qiagen, West Sussex, UK). The concentration and purity of the DNA was determined by UV spectrophotometry. DNA was diluted (1:200) and measured at 260/280 nm in a quartz cuvette (Spectronic® Genesys™ 5, Spectronic Instruments, Leeds, UK). A series of dilutions (10^1, 10^2, 10^3 and 10^4 copies/reaction) of the purified linearised HSV clones were used to evaluate assay sensitivity. Briefly, a standard curve was used to calculate the copy number of the unknown HSV DNA samples. Ten HSV DNA negative samples were tested for PCR inhibition using HSV-1 and HSV-2 positive DNA and primers. Briefly, test samples were 'spiked' into a PCR reaction containing HSV DNA. The performance of the PCR was monitored by quantitative real-time PCR. The mean cycle threshold (C_t) and the standard deviation of the controls were calculated. Samples, in which the mean C_t of the test sample fell outside the mean Ct plus three standard deviations of the controls, were considered to be inhibitory.

2.6 DNA SEQUENCING

2.6.1 Target regions of the HSV genome

Sequencing was performed on two regions of the HSV genome. Target regions included the DNA polymerase and the HSV-2 UL14 gene.

2.6.1.1 DNA Polymerase gene

Genital swabs taken from patients with a clinical diagnosis of genital herpes were processed and stored as previously described (Section 2.1.1). DNA extraction was performed using QIAamp DNA mini kit (Qiagen, West Sussex, UK) as mentioned earlier (Section 2.5.1.1). Samples were prepared for the LightCycler PCR as described above but in the absence of hybridisation probes HSV-2 FLU and LCR. The final volume of 20 µl was made up with deionised water. Following PCR amplification, the DNA was purified for sequencing using the QIAquick PCR Purification kit (Qiagen, West Sussex, UK). The procedure entailed adding 5 volumes of buffer PB to 1 volume of the PCR mix, applying this to the QIAquick spin column and centrifuging the column at 20000 × g for 1 min. The filtrate was discarded, 750 µl of buffer PE was added and the column and the column centrifuged at 20000 × g for 1 min. DNA was eluted by adding 50 µl of de-ionised water to the column, incubating at room temperature for 1 min, followed by a centrifugation at 20000 × g for 1 min.

2.6.1.2 HSV-2 UL14 gene

HSV-2 positive genital swabs confirmed through virus culture and PCR (as described earlier) were used to extract HSV-2 DNA using the QIAamp DNA mini kit (Qiagen, West Sussex, UK) as previously described (Section 2.5.1.1). DNA extracts were stored at -80°C and processed using the HotStarTaq DNA polymerase kit (Qiagen, West Sussex, UK). Briefly, 5 µl of 10X PCR buffer, 5 µl 25mM $MgCl_2$, 2 µl of dNTP mix (10mM each), 5 µl of forward (GCTCACTCGCCATCGGGACAGT) and reverse primers (GCCGAGGTGTACAGGGACCAGACC) (1 µM each) (Sigma-Genosys Ltd, Dorset, UK), 0.25 µl of HotStar Taq DNA polymerase (2.5 units/reaction), and 5 µl DNA were added to make a total reaction volume of 50 µl containing de-ionised

water. Prior to sequencing, the DNA products were purified using the QIAquick PCR Purification kit (Qiagen, West Sussex, UK) as previously described (Section 2.6.1.1) and eluted in 50 µl of de-ionised water.

2.6.2 Sequencing reactions

Sequencing reactions for the DNA polymerase gene were performed using the Sanger dideoxy chain termination method with the ThermoSequenase DYEnamic Direct Cycle sequencing kit (Amersham Pharmacia Biotech Inc, Buckinghamshire, UK), using 3 µl of extracted DNA, 1 µl of each A, C, G, T reagent mix, 1 µl of 0.5 µM HSV forward and reverse primers (as described earlier) labelled with CY5.5 and CY5 respectively (Sigma-Genosys Ltd, Dorset, UK). Reactions were performed on a GeneAmp 9700 thermocycler (PerkinElmer Applied Biosystems, Beaconsfield, UK) under the following conditions: 25 cycles of denaturation at 94°C for 5 min, followed by further denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 70°C for 1 min. Formamide loading dye (7 µl) was added, and the DNA denatured at 80°C for 2 min. Sequencing reactions (2 µl) were run on a OpenGene sequencing system (Visible Genetics, Ontario, Canada) using version 3.16 software as described by the manufacturer.

For the HSV-2 UL14 gene, PCR reactions were performed in a Hybaid Multiblock System (0.5 MBS) (Hybaid GmbH, Heidelberg, Germany) under the following conditions: 1 cycle of denaturation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. For negative controls, DNA

specimens were replaced with de-ionised water. When the PCR program was completed, 5µl of each PCR mixture was mixed with 1 µl of loading dye (Bioline, London, UK) and run on a 1.5% agaorose gel in TBE buffer and visualised by ethidium bromide staining. A 1 kb DNA ladder (Bioline, London, UK) was used as a reference to estimate the size of DNA products. Amplicons that were 500 base pairs in size were sequenced.

2.6.3 Sequence analysis

Sequences generated from the HSV DNA polymerase gene were aligned and compared to other HSV sequences (HSV-1 GenBank Accession no. AB070847; HSV-2 GenBank Accession no. AY038367) sequences in the GenBank database to confirm HSV type using BLAST. For the HSV-2 UL14 gene, sequences were analysed using chromas software version 1.45. HSV-2 UL14 sequences were compared to other known HSV-2 UL14 sequences (GenBank Accession no. CAB06774) and aligned using ClustalW software version 1.82 (http://clustalw.genome.ad.jp) and submitted to the Genbank database.

2.7 T-LYMPHOCYTE ASSAYS

2.7.1 Lymphocyte isolation from whole blood

Peripheral blood mononuclear cells (PBMC) were obtained from by density gradient centrifugation using Ficoll-Plaque (Amersham Biosciences, Buckinghamshire, UK) and collected in preservative-free sodium heparin tubes (Sarstedt, Leicester, UK).

Each tube of 7.5 ml whole blood was under layered with 15 ml of Ficoll in a 50 ml polypropylene tube. The tube was centrifuged at 400 × g for 20 min. PBMC were removed from the lymphocyte layer at the interface just below the plasma layer, and harvested into a new 50 ml tube, which was diluted with RPMI 1640 containing 2 mM L-glutamine, penicillin and streptomycin (Sigma, Dorset, UK) to make 50 ml. Cells were centrifuged twice at 150 × g for 15 min. The supernatant was discarded and the pellet retained. Before the third wash step, the pellet was re-suspended in 50 ml RPMI 1640 containing 2mM L-glutamine, penicillin and streptomycin (Sigma, Dorset, UK) and counted by mixing 50 µl of diluted PBMC and 50 µl of trypan blue solution (Sigma, Dorset, UK). The cell suspension (10 µl) was transferred to a disposable counting chamber and phase microscopy was used to distinguish live cells from dead cells.

Following cell counts ($\times 10^6$), the required volume of cells were transferred to a fresh 50 ml tube and diluted with RPMI 1640 up to 50 ml. The cells were spun at 150 × g for 10 min. Supernatant was discarded and the pellet was processed for cryopreservation and the enzyme linked immunospot (ELISPOT) assay.

2.7.1.1 Cryopreservation of lymphocytes

Cells were cryopreserved on the day PBMC were isolated from blood. Cells were processed as described previously (Section 2.7.1), and the pellet re-suspended at 6×10^6 cells/ml in a freezing solution consisting of 90% FBS and 10% dimethylsulphoxide (DMSO) (Sigma, Dorset, UK) which was pre-cooled to 4°C. The cell suspension was then transferred to 2 ml cryogenic vials (Alpha Laboratories,

Hampshire, UK) in 1 ml aliquots. The vials were placed in freezing containers containing isopropanaol (Nalgene, New York, USA) at room temperature, and then transferred to at -80°C. Stocks of cells were transferred to liquid nitrogen chambers at -192°C the following day.

2.7.2 Enzyme linked immunospot (ELISPOT) assay

The IFN- γ ELISPOT was used to measure T-cell immune responses against HSV among HIV-1 infected and uninfected individuals. Fresh PBMC were used for this assay.

2.7.2.1 Plate coating and antigen stimulation

96 well multiscreen filter plates (Millipore, Gloucestershire, UK) were coated with 100 µl of 1 µg/ml primary anti-IFN-γ antibody (1D1K) (Mabtech, Nacka Strand, Sweden) and incubated overnight at 4°C. The antibody was diluted at a ratio of 1: 100 using sterile filtered PBS. The following day, the plates were washed 6 times with 200 µl of filtered sterile PBS containing 0.05% Tween (Sigma, Dorset, UK). Excess wash solution was removed by tapping the plate onto paper towels soaked with 70% ethanol to minimise contamination. Following the wash step, 100 µl of filtered sterile PBS containing 10% Human AB serum (Sigma, Dorset, UK) was added, and the plate incubated at room temperature for 60 min. PBMC in RPMI 1640 containing 20% Human AB serum were added at a concentration of 3×10^5 cells/well. HSV-1 and HSV-2 lysates (Section 2.3) were diluted in RPMI 1640 to make several concentrations (1 µg/ml, 5 µg/ml and 10 µg/ml) of which 100 µl were added to

respective wells of the plate. In addition, 5 µl of phytohaemoglutinin (PHA) (Sigma, Dorset, UK) at 5 µg/ml and 100 µl of tissue culture medium were added to appropriate wells to serve as positive and negative controls respectively. All samples were run in triplicates. The plate was incubated overnight in a humidified atmosphere of 5% CO_2 at 37°C.

2.7.2.2 Colour development

The plate was decanted and washed six times as previously described with 200 µl of filtered sterile PBS containing 0.05% Tween (Sigma, Dorset, UK). Secondary anti-IFN-γ biotynlated antibody (7-B6-1-Biotin) (Mabtech, Nacka Strand, Sweden) was diluted at a ratio of 1: 1000 in sterile filtered PBS, 100 µl of the diluted antibody solution was added to respective wells of the plate and incubated at room temperature for 3 hours. The plate was washed with 200 µl of filtered sterile PBS containing 0.05% Tween as before, and 100 µl of the diluted streptavidin alkaline phosphatase (streptavidin-ALP) (Mabtech, Nacka Strand, Sweden) was added to the wells of the plate. The streptavidin-ALP was diluted in sterile filtered PBS at a ratio of 1:1000. The plate was incubated for 2 hours at room temperature, washed with sterile PBS/0.05% Tween 6 times before 100 µl of substrate (Biorad, Hertfordshire, UK) was added. The substrate was supplied in a kit and was used according to manufacturer's instructions. The plate was left in the dark for colour development to occur (10-30 min) and the reaction was stopped by washing the wells with water. The plate was left to dry at room temperature overnight before spots were counted using a Nikon stereomicroscope and an automated ELISPOT reader (Karl Zeiss–Imaging Associates, Germany).

2.7.3 Intracellular cytokine staining assay using flow cytometry

Fresh blood was used to isolate PBMC from healthy HSV seropositive and seronegative donors as described earlier (Section 2.7.1). PBMC (1×10^6 cells in 1 ml RPMI 1640 medium containing 10% FCS) were stimulated with 500 µl of infected cell lysate preparations of HSV-1 (1 µg/ml) and HSV-2 (1 µg/ml). To positive and negative controls, 5 µl of staphylococcal enterotoxin B (1 µg/ml) (Sigma, Dorset, UK) and 200 µl of uninfected vero cell extracts were added respectively. Incubation was performed in the presence of 10 µl of anti-CD28 antibody (0.5 µg/ml) (BD Biosciences, Oxford, UK) for co-stimulation. After a 2 hour incubation at 37°C, 1 µl of GolgiPlug (1 µg/ml) (BD Biosciences, Oxford, UK) containing brefeldin A was added to block the transport of cytokines to the cell surface. Following 14 hour incubation at 37°C, the reaction was quenched by washing cells with 2 ml PBS/0.01% azide/0.5% bovine serum albumin.

Centrifugation was performed at 1500 rpm for 5 min, supernatant discarded and pellet re-suspended in 1 µl anti CD3 APC (BD Biosciences, Oxford, UK) 5 µl of cytokine and cell surface marker CD4-peridinin chlorophyll A protein (PerCP) (BD Biosciences, Oxford, UK). The cell suspension was vortexed and stained in the dark for 15 min at 25°C. Cells were washed as before, fixed with 100µl FACS permeabilisation buffer Reagent B and incubated for 15 min at 25°C in the dark. Following permeabilisation, cells were vortexed, 5 µl anti CD69 PE (BD Biosciences, Oxford, UK) and 5 µl anti-human IFN γ-FITC (BD Biosciences, Oxford, UK) was added to detect activated T-cells. Cells were washed as described earlier, fixed in 4%

paraformaldehyde (BD Biosciences, Oxford, UK) and stored in the dark at 4°C before use.

Samples were analysed by a FACScan flow cytometer (Becton Dickinson). Dead cells and debris was excluded by forward and side scatter gating. In the flow cytometry analysis, the CD4 expression of T-cell activation marker CD69 was calculated by gating around the $CD3^{+}CD4^{+}$ cell population on a forward versus side scatter dot plot and 10,000 events were collected for each sample. Dead cells and debris was excluded by forward and side scatter gating. Data was analysed using CellQuest software (Beckton Dickinson, Oxford, UK) and Flow Jo software (Tree Star, Californa, USA).

3. Chapter 3a. The establishment of optimal diagnostic methods for genital herpes: Direct Virus Detection Methods

3.1 INTRODUCTION

In 2004, over 18,500 new cases of genital herpes were reported from GUM clinics in the UK, a 15% increase compared to 1995. These figures underestimate the true prevalence of symptomatic infections as atypical presentations are common and often misdiagnosed (Corey, 1994). Clinical history alone may not be sufficient to provide an accurate diagnosis of genital herpes and laboratory confirmation is required to both confirm and exclude the diagnosis (Mullan & Munday 2003; Cowan *et al.*, 1994). The laboratory diagnosis of genital herpes relies on both direct and indirect virus detection techniques. The main direct methods include virus isolation in cell culture and detection of the viral nucleic acid by molecular methods. Methods based on the demonstration of viral antigens by immunoassays are also available. Indirect methods are based on the detection of virus-specific antibodies.

3.1.2 Virus culture

HSV isolation in cell culture can be performed in a variety of cell lines, including Vero, Hep-2, human diploid, rabbit kidney, and human amnion. HSV positive cultures demonstrate a cytopathic effect (CPE) characterised by cytopathological changes such as syncytia and multinucleated giant cell formation (Ustaçelebi, 2001). Appearance of a characteristic CPE usually takes 3-4 days but may take up to 7-10 days. HSV

isolates obtained by virus culture can be typed as HSV-1 or HSV-2 by immunofluorescence (IF), which utilises fluorescent antibodies directed to HSV type-specific antigens. Virus isolation in cell culture is used routinely for the laboratory diagnosis of HSV infection and is considered the gold diagnostic standard (Ashley, 1993). According to a survey of GUM consultants, in 1999 virus culture was the diagnostic method of choice for genital herpes in the UK (Scoular & Kinghorn, 1999). A more recent survey conducted in 2003 among 25 diagnostic laboratories in England, Wales, Scotland and Northern Ireland showed that virus culture remains the diagnostic method of choice in the large majority of laboratories in the UK (Geretti & Brown, 2005).

Virus culture has several drawbacks. It is slow, labour intensive, and requires up to 7-10 days for the appearance of cytopathic changes in cultured cells or the release of a negative result. Although specificity of cell culture is virtually 100%, levels of virus shedding, quality of specimen and transport conditions influence its sensitivity (Wald *et al.,* 2003; Corey *et al.*, 1983; Scoular, 2002). The rate of virus recovery has been shown to decline significantly with time since the onset of lesions, from 52-93% for vesicles to 41-72% for ulcers and 19-27% for crusted lesions (Corey *et al.*, 1983; Scoular, 2002). The lack of specimen refrigeration after collection and during transport markedly reduces virus viability. This is supported by evidence indicating a 50% reduction in HSV isolation rates during summer months (Wald *et al.*, 2003).

3.1.3 HSV DNA detection by PCR

Molecular techniques can directly detect and type HSV in a wide variety of clinical samples (Volpi & Pica, 1998). The introduction of PCR represented a major breakthrough in the diagnosis of HSV infections of the central nervous system. In patients with HSV encephalitis (HSE), virus isolation from brain biopsies was required for a definitive diagnosis, as cultures of cerebrospinal fluid were positive in only 4% of adult patients (DeBiasi & Tyler, 1999). In a landmark study, CSF was obtained from patients with clinical disease indicative of HSE who underwent diagnostic brain biopsy. HSV DNA was detected by PCR in CSF of 53 (98%) of 54 patients with biopsy-proven HSE. In addition, positive PCR results were found in 3 (6%) of 47 patients whose brain tissue was culture-negative (Lakeman & Whitley, 1995). As a result, HSV DNA detection by PCR supplanted brain biopsy as the modality of choice for diagnosing neurological HSV disease, becoming the current diagnostic method of choice (Cinque *et al.*, 1996).

Although the traditional PCR methodology has previously been applied to the detection of HSV in various clinical specimens, the technique is relatively cumbersome and prone to contamination (Aldea *et al.*, 2002). Recently, real-time PCR assays have been developed for the detection of viruses in clinical specimens. Real-time PCR assays allow rapid virus detection in a closed amplification and detection system, with high sensitivity, low risk of contamination, and no post-amplification steps. Among available assays, the LightCycler PCR technology (Roche Diagnostics, UK) uses two specially designed sequence-specific oligonucleotide probes labelled with fluorescent dyes. One probe carries a fluorescein label (FLU) at

its 3' end and the other a LC Red (LCR) label at its 5' end and a phosphate at its 3'end. Both probes hybridise to the amplicon in close proximity allowing energy transfer to occur between fluorophores (Fluorescence Resonance Energy Transfer) resulting in light emission, measured by the LightCycler. The amount of fluorescence measured is proportional to the amount of specific amplicon generated during the ongoing PCR process.

The melting temperature (Tm) is defined as the temperature at which 50% of the DNA is single-stranded. The most important criteria that determine the Tm are the G+C content and the length of the amplified DNA. The LightCycler monitors the fluorescence continuously, while raising the temperature gradually. When the temperature in the capillary reaches the Tm of the amplified DNA, a sharp decrease in fluorescence occurs as one of the two hybridisation probes can no longer bind to the target sequence. When the method is applied to the detection of HSV DNA, melting curve analysis demonstrates a different Tm for HSV-1 and HSV-2, allowing direct typing of the amplified product.

3.1.4 Study objectives

In order to develop the optimal tools to investigate the epidemiological features of genital herpes, we established and evaluated the performance of a real-time PCR assay on a LightCycler platform for HSV detection in genital swabs. As specimen preparation is a time-consuming process in PCR, evaluation of different specimen preparation methods was necessary in terms of cost, labour and performance. Assay

reliability was evaluated by repeat testing, comparison with other PCR assays and HSV sequence analysis.

3.2 METHODS

3.2.1 Study population and sampling

Genital swabs were collected from 233 GUM clinic attendees from Kings College Hospital, London who presented with clinical features suggestive of genital herpes. The first part of the study comprised genital swabs from 145 consecutive patients. The patients' demographic and clinical data were recorded, including swab site, type of presentation (first or recurrent episode), duration of onset (<5 days or ≥5 days), presence of genital ulceration, administration of antiviral drugs before disease onset (acyclovir, valacyclovir, or famcyclovir), and HIV status. In the second part of the study, 88 additional genital swabs from GUM patients from Kings College Hospital, London were tested to provide a validation group for the larger subset. Swabs were placed in 2.5 ml of supplemented VTM (Section 2.1.1)

3.2.2 Virus culture

Swabs in VTM were vortexed and 200 µl were inoculated in two tubes containing Vero cells. Cultures were maintained as described earlier and examined daily for the appearance of a CPE (Section 2.2). The infected cells were harvested and HSV was typed by direct immunofluorescence (Section 2.2.1).

3.2.3 Specimen preparation for PCR

With a subset of 140 samples, three different methods were used to prepare DNA from 200 µl of specimen in VTM: (1) manual DNA extraction with the QIAamp DNA Mini Kit (Qiagen, West Sussex, UK); (2) automated DNA extraction with the MagNA Pure LC (Roche Diagnostics, East Sussex, UK), and (3) DNA precipitation with polyethylene glycol (PEG) and sodium chloride (NaCl). The QIAamp DNA Mini Kit, MagNA Pure LC instrument, and PEG precipitation methods were employed as described earlier (Section 2.5.1.1, Section 2.5.1.2, Section 2.5.1.3).

Real time PCR was performed with the LightCycler (Roche Diagnostics, East Sussex, UK) using previously described HSV forward and reverse primers and two fluorescently labelled hybridization probes (Section 2.5.2.1). For the PCR, primers were chosen within a highly conserved region of the DNA polymerase gene, yielding a 215 base-pair fragment from each of the HSV-1 and HSV-2 DNA polymerase genes. Following PCR amplification (Section 2.5.2.1), the Tm of DNA fragments and hybridisation probes was used to differentiate between HSV types. The probe Tm was 59°C for HSV-1 and 64°C and/or 71°C for HSV-2. The commercially available RealArt HSV1/2 LC PCR kit (Artus Biotech, Hamburg, Germany) was used to compare the results of 15 specimens.

3.2.4 DNA sequencing

Samples were prepared for LightCycler PCR as described earlier but in the absence of hybridisation probes HSV-2 FLU and LCR (Section 2.6.1.1). Following amplification, the DNA was purified using QIAquick PCR Purification. DNA sequencing was performed by the Sanger dideoxy chain termination method as described earlier using extracted DNA, A, C, G, T reagent mix, HSV forward and reverse primers labelled with CY5.5 and CY5 respectively as described previously (Section 2.6.2). Reactions were performed on a thermocycler and automated DNA sequencing reactions were run on a sequencing system. Sequence data were compared to other HSV sequences present in the GenBank database to confirm HSV type using BLAST followed by sequence alignment using ClustalW version 1.81.

3.2.5 Data analysis

The HSV detection rates for virus culture versus PCR according to clinical characteristics were compared by the χ^2 or Fisher's exact test. To evaluate and compare the three PCR specimen preparation methods, a definition of "true positive" specimen was required. True positive specimens were considered as those that were either positive in both virus culture and PCR, or were virus culture negative but PCR positive in >2 separate assays and by >2 specimen preparation methods. To determine the level of agreement between the three specimen preparation assays, Kappa values were calculated.

3.3 RESULTS

3.3.1 Establishment of HSV Real-time PCR on a LightCycler platform

The PCR method selected amplified a 215 bp product of HSV DNA polymerase gene and allowed its qualitative detection and typing. HSV-1 and HSV-2 positive DNA and deionised water were used as positive and negative controls respectively. Quantification of 5 specimens was carried out by the amplification and purification of known HSV positive DNA from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK). The target gene was directly cloned into the pGEM T easy vector and transformed into JM109 competent cells using the PGEM®-T Easy Vector System (Promega, Southampton, UK) cloning kit (Promega, Southampton, UK). The plasmid DNA was extracted from transformed cells using Wizard^{R} *Plus* SV Miniprep DNA Purification System kit (Promega, Southampton, UK). A series of dilutions (10^1, 10^2, 10^3 and 10^4 copies/reaction) of the plasmid standards was used to determine assay sensitivity. Briefly, a standard graph comprising the C_t values from the serial dilutions of the plasmid and the C_t values from unknown samples were plotted to calculate the number of HSV genomes per millilitre. The LightCycler Quantitation Analysis software provided the crossing point (C_p) (i.e. cycle number at which reactions enter the log/linear phase) to determine the initial target concentration.

Standards were tested in duplicate in 10 separate runs to determine the threshold and reproducibility of the real-time PCR assay. As 10 copies per reaction were consistently detected in all of the runs for both HSV-1 and HSV-2, this was

considered to be the lowest limit of quantification (data not shown). To check for inhibition, experiments were conducted where 10 HSV negative clinical specimens were spiked with HSV-1 and HSV-2 DNA (5 HSV-1 and 5 HSV-2) and subjected to multiple specimen preparation methods (see below). No significant inhibition was detected in these experiments. To check for assay reproducibility, experiments were performed in triplicates. Results revealed the assay to be highly reproducible with all three specimen preparation methods accurately exhibiting equivalent typing sensitivity in all 10 specimens.

3.3.2 Comparison of specimen preparation methods

Three specimen preparation methods using manual DNA extraction with the QIAamp DNA mini kit, automated DNA extraction with MagNA Pure LC, and virus precipitation with PEG were compared to determine assay performance, labour intensity and reagent cost. Of 140 specimens being compared, 120/140 (86%) were either HSV negative or positive by all three methods, 12/140 (8%) were positive by two methods and 8/140 (6%) were positive by only one method. In addition, mean crossing point values were highly concordant in HSV positive specimens between the three PCR preparatory methods. Of the 8 specimens that were positive by only one method, three were also virus culture positive and therefore scored as true positive. According to the definition of true positive given above, HSV detection rate was PEG precipitation > MagNA Pure LC > QIAamp DNA mini kit (Table 3.1). A high level of agreement was observed between the three preparatory assays, with Kappa values ≥ 0.84.

HSV was detected in 65/72 (90%) true positive specimens by PEG precipitation, including three specimens that were positive by culture but negative by QIAamp DNA mini kit and MagNA Pure LC. In comparison to the other preparation methods, PEG precipitation was also faster and least expensive (Table 3.1).

Table 3.1 Comparison of detection rate, duration and cost of three specimen preparation methods with virus culture

	DNA extraction		Virus	Virus culture
	Manual	Automated	precipitation	
Relative detection rate[a]	61/72 (85%)	62/72 (86%)	65/72 (90%)	42/72 (58%)
Duration				
Specimen preparation	90 m	120 m	60 m	-
PCR	81 m	101m	81m	-
Total[c]	171m	221m	141m	2-10 days
Reagent cost per test (£):	3.99	9.27	2.44	Negative sample: 1.00 Positive sample[b]: 1.55

[a]True Positive: specimens that had tested HSV positive in both virus culture and PCR, or if virus culture negative, positive in ≥2 separate PCR assay and by ≥2 specimen preparation methods; [b]Typed by direct immunofluorescence; [c]Time taken to process 1 sample.

3.3.3 HSV typing

HSV typing was performed using LightCycler melting curve analysis to differentiate between HSV types. Melting curve analysis was based on base-pair (bp) differences between HSV-1 and HSV-2 amplicons in the probe-binding region of the DNA polymerase gene. The probe melting temperature was 59°C for HSV-1 and 64°C and/or 71°C for HSV-2 (Figure 3.1). Among 132 HSV-positive swabs, 106/132 (80%) were typed as HSV-2 and 12/132 (9%) as HSV-1. Results were all confirmed by repeated testing. Samples that yielded an isolate in virus culture also showed consistent results of HSV typing by Immunofluorescence. In addition, 15 specimens were tested with a commercial assay with identical HSV typing results. A further 14/132 HSV positive swabs showed atypical melting curves with Tm >60°C (Table 3.2).

3.3.4 Characterisation of specimens with atypical melting curves

Overall 6/14 (43%) specimens showed ≥2°C variation from the HSV-2 reference Tm (64°C and/or 71°C) (Table 3.2). The most problematic samples to interpret were those with a Tm below 64°C and close to the reference Tm for HSV-1 (59°C). To determine whether atypical melting curves were a result of target sequence variation, the corresponding DNA polymerase region was sequenced. All 14 samples were typed as HSV-2 following DNA polymerase gene sequence alignment. In addition they were typed as HSV-2 by immunofluorescence testing of virus isolates. Sequence variation in the 100 bp region of the FLU probe binding site of the DNA polymerase gene was

evident in 4/14 specimens with Tm ranging between 61°C - 66°C (Table 3.2). No sequence variation was observed in the target HSV-2 sequence among the 10 remaining specimens.

Table 3.2 Nucleotide sequences of HSV positive specimens with Tm >60°C

Specimen	HSV-2 Sequence
HSV-2 Control	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
Probe LCR	GGCGTCATCTGCGGGGGCAAG
Probe FLU	GCTCATCAAGGGCGTG-GATCTG-GTGCGC
1	CGGCGTCATCTGCGGGGGCAAGATGCTCATTAAGGGCGTC-GACCTG-GTGCGCAAAAAC
2	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
3	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
4	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
5	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
6	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
7	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
8	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
9	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
10	CGGCGTCATCTGCGGGGGCAAGATGCTCATTAAGGGCGTC-GACCTG-GTGCGCAAAAAC
11	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
12	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTG-GTGCGCAAAAAC
13	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTGAGATCTG-GTGCGCAAA---
14	CGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTG-GATCTGTGTGCGCAAAAAC

Probes are boxed and nucleotide differences are shaded.

To determine whether different specimen preparation methods might influence the outcome of the HSV melting curve, the 14 specimens were re-processed with QIAamp DNA mini kit, automated DNA extraction with the MagNA Pure LC and viral precipitation with PEG. Repeat testing with 6/14 samples showed reproducible Tm values with the three specimen preparation methods (Table 3.3). Of samples with ≥2°C variation from the reference Tm, identical Tm were observed in 4 QIAamp DNA mini kit and MagNA Pure LC, 1 in QIAamp DNA mini kit and PEG, and 1 in MagNA Pure LC and PEG preparations. Overall, discrepancies were fewer in specimens processed by MagNA Pure LC or PEG precipitation compared with the QIAamp DNA mini kit. Overall, between all three specimen preparation methods, 7 samples had atypical Tms, of which 5 specimens had conserved sequences and 2 had sequence differences at the probe binding site.

Table 3.3 Comparison of melting temperatures (Tm,°C) of HSV-2 positive swabs following three specimen preparation methods and parallel testing in the same LightCycler PCR assay. The reference Tm for HSV-2 was 64°C and/or 71°C

Specimen preparation methods		
Manual DNA extraction: QIAamp DNA mini kit	Automated DNA extraction: MagNA Pure LC	Virus precipitation: Polyethylene glycol/NaCl
61*†	60†	61
62*	61	61
65*	64	65
65†	63†	68†
66*†	66†	64†
66	66	66
66	66	71
66†	66†	65†
65, 71†	66, 71†	62, 72†
72†	71†	65†
72	71	71
72	71	71
72	71	71
72	72	66
Total samples with Tm variation		
14	9	9
Total samples with Tm variation ≥2°C		
6/14	6/9	5/9

†The Tm value was replicated in 6 independent experiments for each specimen preparation method; *sequence variation in probe binding region of the DNA polymerase gene

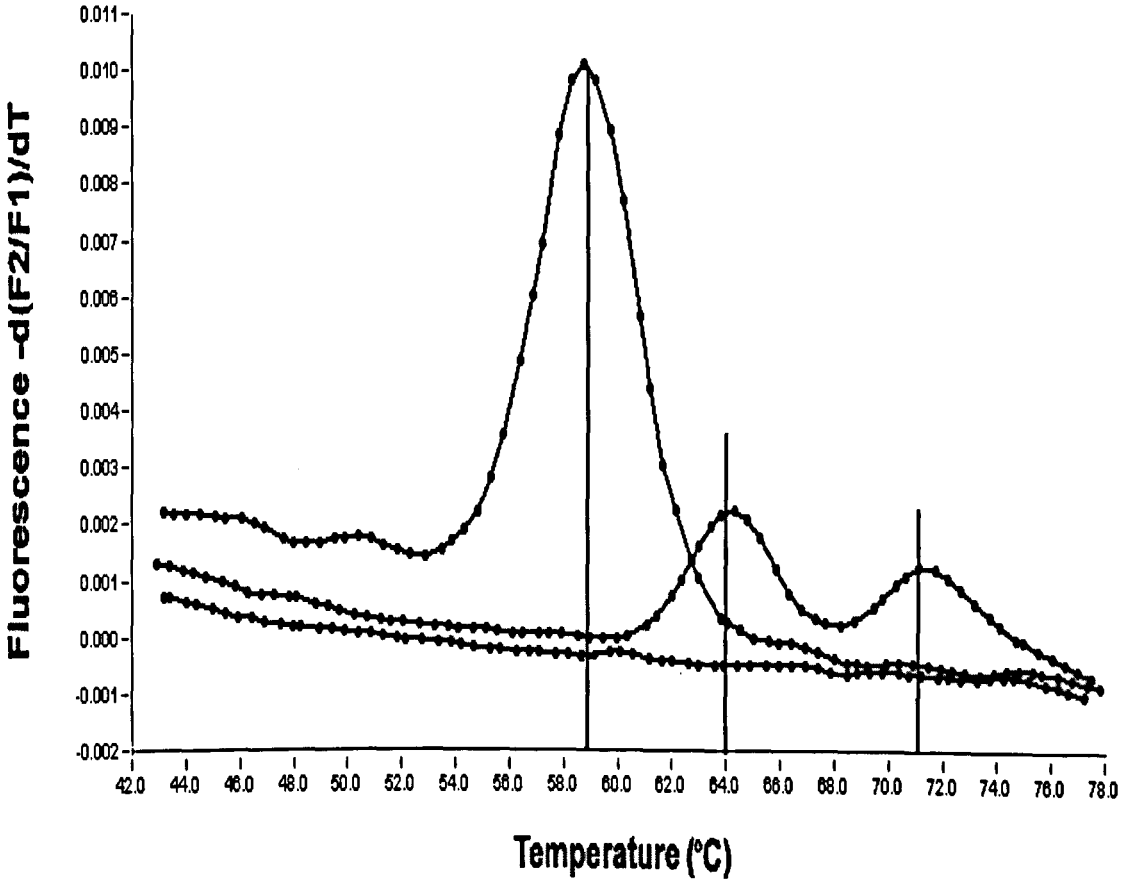


Figure 3.1. Typing of HSV-1 and HSV-2 by melting curve analysis.

3.3.5 Comparison of virus culture and PCR

We tested genital swabs from 145 patients with a clinical diagnosis of genital herpes using virus culture and PCR. The most common swab sites were the penile skin in males (63/85, 74%) and the vulva in females (48/60, 80%) (Table 3.4). Among patients with visible genital ulceration, a significant difference in detection rate was observed by both virus culture and PCR (34% vs. 64%). However in the absence of visible ulceration no significant differences between virus culture and PCR were observed (25% vs. 31%). By virus culture and PCR, 45/145 (31%) and 77/145 (53%) specimens were HSV positive respectively. All HSV negative specimens by culture (n=32) were confirmed HSV PCR positive upon retesting in ≥2 independent replicate PCR assays (mean 2.75 assays per sample). In addition a random subset of 15 samples, were also confirmed HSV positive by The RealArt™ HSV1/2 LC PCR kit (Artus Biotech, Hamburg, Germany). Real-time PCR was shown to increase HSV detection by 71% (32/45). No patient with positive culture had a negative PCR. A positive virus culture result was obtained in 3 days (range 2-10 days) with negative results being available in 10 days, compared to less than 4 hours for PCR results.

Table 3.4 Comparison of virus culture and PCR for the detection of HSV in different clinical presentations of genital herpes

	Total	HSV positive n (%)	
		Virus culture	PCR
First episode	83	26 (31)	41 (49)
Recurrent episode	62	19 (31)	36 (58)
Onset < 5 days	64	27 (42)	38 (59)
Onset ≥ 5 days	81	18 (22)	39 (48)
Visible ulceration	97	33 (34)	62 (64)
No visible ulceration	48	12 (25)	15 (31)
HIV positive	17	7 (41)	12 (71)
HIV negative	58	20 (34)	32 (55)
HIV status unknown	70	18 (26)	33 (47)
Males	85	24 (28)	43 (51)
Females	60	21 (35)	34 (57)
Total	145	45 (31)	77 (53)

3.3.6 Performance of virus culture according to demographic and clinical characteristics

Virus culture was more frequently positive in patients presenting <5 days of onset of symptoms (27/64, 42%) than in those who presented later (18/81, 22%, p=0.01). The proportion of patients with a positive culture was similar in first episode and recurrent disease.

3.3.7 Performance of HSV PCR according to demographic and clinical characteristics

HSV detection rates were similar among both sexes, with PCR increasing detection by 79% (19/24) and 62% (13/21) in men and women respectively (Table 3.4). No significant differences were observed among patients who presented < 5 days or ≥ 5 days of onset of symptoms with a positive PCR result (38/64, 59% vs. 39/81, 48%, p=0.2). As a result, PCR increased HSV detection by 41% (11/27) in early disease and 116% (21/18) in late disease. PCR increased HSV detection by 58% (15/26) in first disease and 89% (17/19) in recurrent disease. Detection of HSV in the presence of f genital ulcers was also enhanced by PCR (Table 3.4). Improved detection by PCR was seen regardless of HIV status.

3.3.8 Validation study

To validate the previous results, the study was further extended to include an additional 88 consecutive genital swabs from patients with suspected genital herpes. Of these, 34/88 (39%) were virus culture positive and 55/88 (63%) were PCR positive. In this additional subset of specimens, PCR increased HSV detection rate by 62% (21/34) validating the data from the larger subset. Overall in our entire cohort, 34% (79/233) of swabs were HSV positive by culture and 132/233 (57%) were positive by PCR.

3.4 DISCUSSION

Virus culture remains the reference method for the laboratory diagnosis of genital herpes in the UK (Geretti & Brown, 2005) despite its limitations (Scoular, 2002; Wald *et al.,* 2003). Virus culture is a labour-intensive and time consuming methodology requiring stringent conditions for specimen transport and storage conditions to preserve virus viability (Wald *et al.,* 2003).

In this study we demonstrated that real-time PCR significantly increased the rate of HSV detection in genital swabs by 61-71% compared to virus culture. Among patients with visible genital ulcerations, 88% more infections were detected by PCR than virus culture. This might be due to replicating virus on the mucosa. During HSV reactivation, the virus is released from the nerve endings in the sub-mucosa. Therefore breakages in the epithelial barrier would result in virus transfer from the sub-mucosa to the mucosa (Wald *et al.,* 2003), leading to increased detection sensitivity. A recent study and 12 patients (10%) among In the absence of PCR testing, 53/233 of the symptomatic patients would have received a negative result, thus preventing appropriate counselling on prognosis and risk of transmission and possibly triggering additional clinic visits and investigations. Real time PCR exhibited increased sensitivity for the detection of both HSV-1 and HSV-2 relative to virus culture. Detection sensitivity of 10 copies/reaction in the proficiency evaluation along with the high concordance in replicate tests confirmed the high sensitivity and specificity of the assay.

The performance, labour and cost of HSV PCR for HSV detection in genital swabs was also investigated by comparing three specimen preparation methods. Kappa values of ≥0.84 indicated a high level of agreement between the three preparatory assays. Virus precipitation by PEG, although not previously used for LightCycler PCR, had the highest detection rates, was the least expensive and required the shortest preparation time, thus providing a valid option for specimen processing prior to PCR. This study led to the first published evidence that PEG precipitation offers a valid method for specimen preparation from genital swabs prior to PCR.

Even though PCR shows increased sensitivity over virus culture, its implementation may be limited by concerns of contamination. The advantages of real-time PCR are that it allows direct quantification of the target and avoids post–PCR handling, leading to minimal contamination risk with PCR products. Retesting of HSV positive specimens yielded reproducible results validating the assay to be reliable. In addition, the high prevalence of HSV disease in our cohort could have contributed to assay reproducibility (Van Doornum *et al.,* 2003). Although the costs of consumables for PCR (£2.44-£9.27) were higher than those for virus culture (£1.00-£1.55), real-time PCR proved cost-efficient by significantly reducing labour cost per sample.

Melting curve analysis allows the differentiation between HSV types, characterised by different Tm values for HSV-1 and HSV-2 specific probes. Real-time PCR allowed for the typing of HSV without additional, time consuming post-PCR steps. Sequencing of a subset of HSV-2 positive samples showed a low prevalence of sequence variation indicating robustness and reliability of the assay. In a subset of samples, atypical melting curves were observed that did not conform to the expected

Tm for HSV-1 and HSV-2. Atypical melting curves have been previously reported as a result of sequence variation causing base-pair mismatches over the probe sites (Anderson *et al.*, 2003; Issa *et al.*, 2005). In our study, of the 14 specimens sequenced, 4/14 (29%) showed sequence variation in the FLU probe-binding site whereas 10/14 (71%) specimens did not reveal sequence differences. Differences in Tm were shown to arise from different specimen preparation methods. Fewer discrepancies were observed in specimens prepared by the MagNA Pure LC or the PEG precipitation compared with the QIAamp DNA mini kit. This suggested that atypical melting curves may occur as a result of factors other than sequence variation. These may include carryover of contaminants, such as salts during purification, which may influence the annealing stringency of the probe/target complex. Thus multiple factors including, but not limited to sequence variation may complicate melting curve analysis, following real-time LightCycler PCR. These findings indicated that differentiation between HSV types may require alternative typing procedures for a small subset of samples with atypical melting curves.

In this study, the increased HSV detection by PCR was observed in different clinical presentations. Previous reports have shown virus culture to yield a significant higher HSV detection rate among patients with first episode disease (84%) compared to those with HSV recurrences (42%) (Corey & Holmes, 1983). In contrast, no difference in HSV detection rate by virus culture was observed in first (26/83, 31%) and recurrent disease (19/62, 31%) in this study. However, HSV detection rates were higher in persons who presented < 5 days onset of symptoms (27/64, 42%) than in those who presented later (18/81, 22%), indicating an effect of declining virus shedding on assay sensitivity. HSV detection by PCR was higher than virus culture

(18/81, 22% vs. 39/81, 48%) among patients who presented late after the onset of symptoms (≥ 5 days). This finding suggests that declining levels of virus shedding in older lesions favour PCR as the more the sensitive method. Even among patients who presented <5 days of onset of symptoms, detection was significantly increased by PCR (38/64, 59%) compared with virus culture (27/64, 42%). As our study was performed during the summer months, warm conditions may have affected HSV viability and influenced on the sensitivity of virus culture. This explanation is consistent with findings from a recent study which showed virus viability in 2116 genital swabs to decrease by approximately half in summer compared to winter (Wald *et al.*, 2003).

Previous studies that investigated over 60000 mucocutaneous swabs by virus culture and PCR have also reported increased sensitivity of PCR over virus culture (Table 3.5). Overall, PCR has shown to be 25-89% more sensitive than virus culture (Scoular *et al.*, 2002; Burrows *et al.*, 2002; Aldea *et al.*, 2002; Van Doornum *et al.*, 2003; Wald *et al.*, 2003; Schmutzhard *et al.*, 2004).

Only a small proportion of laboratories in the UK were shown to use PCR for processing genital swabs, despite the wide availability of PCR for HSV DNA detection in CSF. A survey of diagnostic methods for HSV detection demonstrated 17/25 (68%) laboratories used HSV PCR for processing of CSF specimens (Geretti & Brown, 2005). This contrasted with only 5/25 (20%) providing HSV PCR for genital swabs. The findings presented here, together with evidence from other investigators, provide strong support to the use of PCR for the diagnosis of genital HSV infections. Laboratory investigations provide important guidance for the management of patients

with suspected genital herpes. Although virus culture continues to be the reference diagnostic test, our results showed that its ability to detect HSV in a large proportion of infected individuals was clearly deficient compared to PCR. The findings strongly supported the implementation of PCR to investigate the epidemiological features of genital herpes.

Table 3.5 Overview of studies that compared the diagnostic yield of virus culture and PCR for HSV detection in mucocutaneous swabs

Swab type	No	% VC+	% PCR+	Specimen preparation	PCR method	Typing	Reference
Genital	236	37	41	Manual	Real-time	RFLP	Scoular *et al.*, 2002
Genital, dermal	668	11	14	Automated	Real-time	Yes	Van Doornum *et al.*, 2003
Genital	28	64	100	Manual	Real-time	Yes	Aldea *et al.*, 2002
Genital	63	57	99	Manual	Real-time	Yes	Burrows *et al.*, 2002
Genital	372	35	48	Manual	Real-time	Yes	Bruisten *et al.*, 2001
Genital, dermal	88	78	100	Manual	Real-time	Yes	Espy *et al.*, 2000
Genital	36471	3	12	Manual	Real-time	No	Wald *et al.*, 2003
Genital	100	32	36	Manual	Real-time Multiplex	Yes	Marshall *et al.*, 2001
Genital, dermal	110	49	62	Manual	Real-time	Yes	Schmutzhard *et al.*, 2004
Genital	194	48	59	Manual	Gel-based	RFLP	Slomka *et al.*, 1998
Genital, dermal	89	50	64	Manual	Real-time	Yes	Filen *et al.*, 2004
Cutaneous	246	47	60	Manual	PCR-EIA	Yes	Safrin *et al.*, 1997
Genital, dermal	479	45	45	Automated	Real-time	Yes	Namvar *et al.*, 2005
Genital	24345	2	13	Manual	Real-time Multiplex	Yes	Corey *et al.*, 2005
Genital, dermal	226	9	14	Automated	Real-time	Yes	Mengelle *et al.*, 2004
Genital, dermal	182	27	39	Automated	Real-time	Yes	Stránská *et al.*, 2004
Genital, dermal	198	35	48	Manual and Automated	Real-time	Yes	Espy *et al.*, 2001
Genital, dermal	104	41	53	Manual	Real-time	Yes	Koenig *et al.*, 2001
Genital	233	34	57	Manual and Automated	Real-time	Yes	Ramaswamy *et al.*, 2005
Total	64432	704	964				

Chapter 3b. The establishment of optimal diagnostic methods for genital herpes: Antibody detection

3.5 INTRODUCTION

Infection with HSV-1 and HSV-2 can be classified into primary, initial and recurrent on the basis of antibody results. Primary infections with either virus type occur in the absence of pre-existing antibodies to HSV-1 or HSV-2. Initial infections with one virus type occur in the presence of antibodies to the other type. Although the initial immune response to HSV is complex and not completely understood, the first antibodies to be detected are IgM followed by IgG. Several of the viral envelope proteins of HSV-2 (gG, gB, gD, VP5, VP16, ICP35) have been shown to be immunogenic, eliciting an antibody response in humans (Görander *et al.*, 2003; Ashley *et al.*, 1994; Straus *et al.*, 1997; Bellner *et al.*, 2005; Ho *et al.*, 1993). Due to a high degree of genetic similarity between HSV-1 and HSV-2, most viral proteins induce a cross-reactive antibody response. Glycoprotein G of HSV-1 (gG-1) and HSV-2 (gG-2) are the only known viral envelope protein which elicits a type-specific antibody response.

The time required for the development of IgG antibodies following HSV infection varies between 21-42 days, although detectable IgG has been demonstrated among several individuals 21–28 days from the onset of symptoms (Ashley *et al.*, 1998; Slomka *et al.*, 1995; Ho *et al.*, 1993; Page *et al.*, 2003). IgM antibodies are usually

detectable 9–10 days after exposure and last 7–14 days, but may remain detectable for up to 6 weeks in some individuals (Ho *et al.*, 1992; Ho *et al.*, 1993; Ashley, 1998). Therefore, serological testing soon after the first development of symptoms may help to determine if an HSV infection is recently acquired (IgM antibody positive, IgG antibody negative), or pre-existing (IgG antibody positive).

Both HSV-1 and HSV-2 are closely related, with an overall homology of 50% (Dowbenko & Lasky, 1984; Levi *et al.*, 1996). The HSV virion contains 7 glycoproteins (B, C, D, E, G H, and I). There is extensive antibody cross-reactivity between the glycoproteins. However the glycoprotein G (gG) is HSV type-specific (Ashley & Wald, 1999; Levi *et al.*, 1996; Morrow *et al.*, 2004; Liljeqvist *et al.*, 2002). The US4 gene encoding gG is located in the unique short region of the HSV genome. The percentage of HSV-1 and HSV-2 homology in the C terminal region of the protein is higher than that observed in the N terminal region (Levi *et al.*, 1996). In addition, the gG protein of HSV-2 (gG-2) is 460 amino acids longer than its HSV-1 counterpart (gG-1) (McGeoch *et al.*, 1987).

Definitive diagnosis of genital herpes is essential for the management of patients and transmission prevention strategies. In the past, accurate serologic diagnosis of HSV infection was hampered by inaccurate commercial tests that failed to distinguish between HSV-1 and HSV-2 antibodies. The development of IgG and IgM type specific serological tests have aided in the diagnosis, treatment, and counselling of patients presenting with primary genital HSV infection. The western blot assay

remains the current gold-standard test, due to its high specificity and sensitivity. However, this assay is expensive and cumbersome, requiring high expertise, and is thus not widely used. Commercial assays, based on type-specific gG antigens of HSV-1 and HSV-2 have become recently available (Table 3.6). Most of these tests use colorimetric end points based on enzyme-substrate reactions (EIA), although one Immunblot assay is also available. The recently FDA approved HerpeSelect HSV gG diagnostic kits (HerpeSelect 1 EIA, HerpeSelect 2 EIA, HerpeSelect Immunoblot, Focus Technologies, California, USA) have been demonstrated to be very sensitive and specific compared to the Western blot (96-100%), (Ashley, 2001) (Table 3.6).

Table 3.6. Performance of some commercial serological assays based on HSV-2 gG (gG-2)

HSV-2 serological test (Manufacturer)	FDA approved	Sensitivity (%)	Specificity (%)
HSV-2 EIA IgG (Focus Technologies, USA)	Yes	96-100	97-100
HSV-1 and HSV-2 IgG differentiation Immunoblot (Focus Technologies, USA)	Yes	97-100	98
POCkit HSV-2 (Diagnology, Northern Ireland)	Yes	93-100	94-97
Cobas Core HSV-2 IgG EIA (Roche, Switzerland)	No	93	98
Captia Select HSV-2 EIA (Centecor, USA)	No	90-92	91-99

The performance of HSV type specific IgG assays is not affected by HIV-1 status (Laeyendecker *et al.*, 2004). Although many studies from USA and Europe have shown a high degree of concordance for gG-based serologic assays, recent evidence has demonstrated that recombinant gG-2 (rgG-2) EIA (Focus Technologies, California, USA) may give false-positive results in certain African sera (Hogrefe *et al.*, 2002).

The primary objective of this chapter is to establish the use of HSV-type specific serology as a tool to study the epidemiology of genital herpes. As part of the evaluation, we investigated the use of commercially available HSV type-specific antibody tests in various clinical scenarios. We also evaluated the performance of a gG inhibition EIA assay with sera from sub-Sahara Africa.

3.6 METHODS

3.6.1 Antibody testing

Patient sera were tested using the HerpeSelect IgG HSV-1 and HSV-2 EIA (Focus Technologies, Cypress, California, USA) according to manufacturer's instructions. The sample ODs were compared with the reference cut-off readings to determine the index values, which were reported according to the formula [Specimen OD/mean OD of cut-off calibrator].

Samples were also tested by the HerpeSelect Immunoblot IgG (Focus Technologies, Cypress, California, USA) (Section 2.4.4), according to the manufacturer's instructions. Results were read visually as coloured precipitate at different positions for an anti-human serum band, HSV common antigen band, HSV gG-1 band, and a HSV gG-2 band.

To determine the specificity of the HSV-2 EIA, an inhibition-EIA was established, as described in Chapter 2 by pre-incubation patient sera withHSV-1 (MacIntyre strain) and HSV-2 (MS strain) lysates (1 mg/ml). Percent inhibition relative to untreated serum was determined by the formula [1 - (index of HSV-2 lysate well/index of HSV-1 well)] X 100. Samples showing inhibition ≥60% with HSV-2 but not with HSV-1 lysates in two independent assays were scored as HSV-2 positive.

3.7 RESULTS

3.7.1 Performance of the EIA and Immunoblot

We tested 537 serum samples by HSV-2 EIA. Using the manufacturer's recommended cut-off of 1.1 for a positive result and >0.9 to 1.0 for an equivocal result, 316 samples were scored as positive, 79 as equivocal and 142 as negative. Equivocal (>0.9 to 1.0) and positive (>1.1) samples were retested by Immunoblot. 51/60 (85%) EIA positive samples with index values between 0.9 and 3.0 were confirmed positive by the Immunoblot and 9/60 (15%) were negative. Of the 205

serum samples with index value >3.0, 186/205 (91%) were positive by Immunoblot and 19/205 (9%) were negative. The median EIA index value was 3.9 (range 1.2-14.4) for samples that tested positive by Immunoblot, and 1.1 (0.9-4.7) for those that tested negative. Based on these results, we increased the EIA cut-off value for a positive result from 1.1 to 3.1.

3.7.2 Inhibition EIA

Previous studies have highlighted problems of specificity associated with the use of the HerpeSelect EIA with samples from patients from Uganda and Kenya, when using a cut-off of 1.1 for the screening EIA (Hogrefe *et al.*, 2002; Laeyendecker *et al.*, 2004). To address this issue 32 samples that had tested positive by routine EIA (>1.1) were retested using an inhibition-EIA, as described earlier (Section 2.4.3). By inhibition EIA, 20/32 (63%) Ugandan samples and 2/6 (33%) Kenyan samples showed inhibitions levels ≥60%. The median EIA index value for samples that showed inhibition values ≥60% was 8.1 (7.2-17.2) whereas that for samples that showed lower inhibition levels was 2.2 (1.1-3.8). This confirmed that the use of a higher cut-off for the HSV-2 EIA than that recommended by the manufacturers can be used to increase specificity without compromising sensitivity of the assay.

3.7.3 Prospective evaluation of HSV-2 antibody seroconversion

The first set of prospective serum samples was obtained from a woman who developed a first episode of laboratory-confirmed genital HSV-2 infection during pregnancy and transmitted the HSV-2 infection to the neonate. Stored serum samples collected at week 12 of pregnancy and 1 week after delivery were tested retrospectively by the Focus HSV type-specific EIA. No HSV-1 and HSV-2 antibodies were detected in maternal sera at 12 weeks of pregnancy and 1 week after delivery. However, HSV-2 antibodies were detected 6 weeks after delivery, indicating a newly acquired primary HSV-2 infection (Table 3.7). Paternal sera collected 1 week after delivery was HSV-1 seronegative and HSV-2 seropositive, suggesting that the father was the source of the maternal infection. Both parents denied a clinical history of genital herpes.

Table 3.7. HSV type-specific serological follow-up in a couple implicated in the neonatal transmission of genital HSV-2 infection

Time from conception in weeks	Maternal HSV antibody status[a]		Paternal HSV antibody status[a†]	
	HSV-1	HSV-2	HSV-1	HSV-2
12	0.29	0.46	ND	ND
42	0.55	0.42	0.77	7.47
47	0.65	10.82	ND	ND

[a] HSV EIA index values; [†]ND, no data

A second set of samples was obtained from a woman who developed brain stem encephalitis associated with HSV-2 detection in CSF by PCR, and evidence of intrathecal synthesis of HSV IgG antibodies (Tang *et al.*, 2003) (Table 3.8). Sequential serum samples from the patient showed that she remained HSV-1 antibody negative throughout the serological follow-up period of 60 days. The patient was initially HSV-2 seronegative on day 5 of onset, and seroconverted to being HSV-2 positive on day 28. Samples taken on days 45, and 60 were HSV-2 antibody positive with increasing EIA OD values (Table 3.8). Immunoblot analysis on 4 samples (days 5-40) revealed the presence of bands on the positions for anti human serum, HSV common antigen, and gG-2. No band for gG-1 was seen. These findings indicated that the brain stem encephalitis was associated with a primary HSV-2 infection, as indicated by seroconversion to HSV-2 antibodies in the absence of antibodies to HSV-1.

Table 3.8. HSV serological follow-up in a patient with acute HSV encephalitis

HSV EIA[†]	HSV serological follow up since onset of symptoms in days			
	5	28	45	60
HSV-1	0.17	0.31	0.42	0.64
HSV-2	0.39	4.78	8.91	11.63

[†]HSV EIA index values over the 60 days follow-up period

3.7.4 Evaluation in patients with a diagnosis of genital herpes

Serum samples were collected from 70 GUM clinic attendees with suspected genital herpes. Serological testing was performed by the Focus EIA with equivocal results confirmed by an Immunoblot IgG (Focus Technology, USA) (Section 2.4.1, Section 2.4.4). Based on the clinical history and result of HSV-2 PCR of the genital swab, serum samples were classified as either (1) first-episode genital disease and positive HSV-2 PCR, (2) a history of recurrent genital disease and positive HSV-2 PCR, and (3) a history of recurrent genital disease and negative HSV PCR. In total, 32 samples were HSV-2 antibody positive, including four that were equivocal by EIA but positive by immunoblot.

Among patients with first-episode genital disease and a positive HSV-2 PCR, 26/42 (62%) were HSV-2 antibody negative. This finding was consistent with a newly acquired infection. A follow-up to demonstrate seroconversion in these patients was not performed due to the blinded nature of antibody testing. The presence of HSV-2 specific antibodies at the time of presentation in the remaining 16/42 (38%) patients with a positive HSV-2 PCR indicated that the infection was established than a newly acquired infection (Figure 3.2). Among patients with recurrent genital disease and a positive HSV-2 PCR, 9/9 (100%) tested positive for HSV-2 antibodies. This was consistent with the clinical diagnosis of recurrent disease. Among patients with a history of recurrent genital disease but negative HSV PCR, 7/19 (37%) had HSV-2

antibodies, indicating that genital herpes was the likely cause of recurrent symptoms (Figure 3.2).

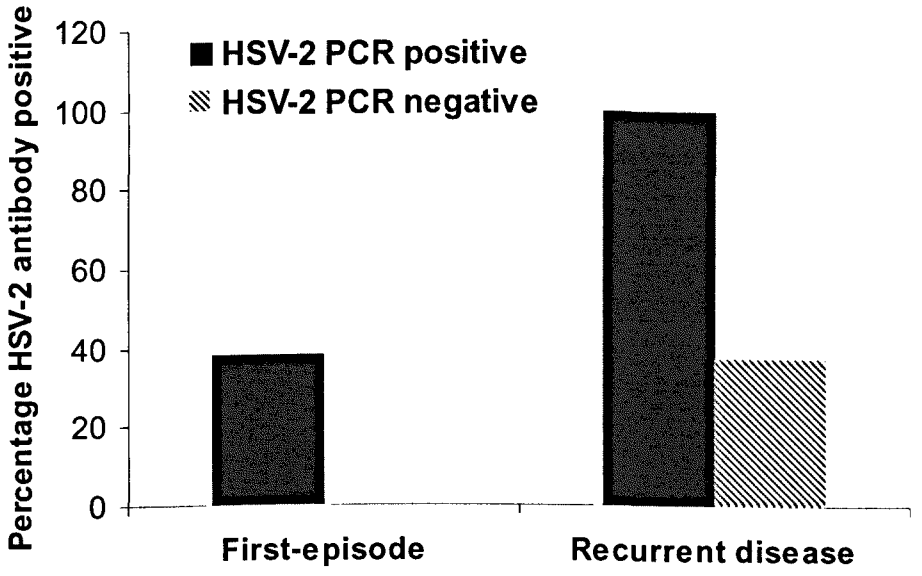


Figure 3.2. Detection of HSV-2 specific antibodies in patients with first-episode and recurrent genital disease, stratified according to the results of HSV-2 DNA detection in genital swabs using polymerase chain reaction (PCR). There were 42 patients with first-episode disease and positive HSV PCR, 9 patients with recurrent genital disease and positive HSV PCR, and 19 patients with a history of recurrent genital disease but negative HSV PCR.

3.8 DISCUSSION

We evaluated the performance of 2 commercial serological assays (HerpeSelect EIA and HerpeSelect Immunoblot), and an in-house inhibition EIA. Overall there was excellent concordance between the EIA and Immunoblot results, which is to be expected given that tests employ a similar antigenic format. One study which compared and evaluated the performance of the HerpeSelect Immunoblot reported that the HerpeSelect Immunoblot had specificity, sensitivity, negative and positive predictive values of 94%, 98%, 97% and 96.5% respectively (Ribes *et al.*, 2002). Another study demonstrated 100% concordance between the HerpeSelect Immunoblot and the HerpeSelect EIA in a study of 506/532 concordant specimens (Ribes *et al.*, 2002). Nonetheless optimal correlation was found when the cut-off was raised from 1.1 to >3.0, which was associated with a confirmed Immunoblot reactivity in 91% of samples. Since the median EIA index value was 3.9 (range 1.2-14.4) for samples that tested positive by Immunoblot, and 1.1 (0.9-4.7) for those that tested negative, a cut-off value of 3.1 was chosen in order to improve specificity while preserving good sensitivity.

When using an inhibition EIA, discrepant results were obtained with specimens from patients from Uganda and to a lesser extent Kenya. The evaluation clearly indicated that the positive cut-off value for the EIA should be raised from the 1.1 recommended by the manufacturer. The median EIA index value for samples that tested positive by inhibition EIA was 8.1 (7.2-17.2) whereas that for samples that tested negative was

2.2 (0.9-3.8). Thus a cut-off >3.8 may be considered to ensure optimal specificity of the assay with some samples from Kenya and Uganda. To ensure no loss of sensitivity and given that the origin of samples was not always available, in subsequent studies we adopted the same cut-off of 3.1 for all samples. This is in line with recent recommendations (Laeyendecker *et al.*, 2004).

One study that investigated the performance of the HSV-2 EIA on Ugandan sera, reported an increase in the index cut-off value to 3.4 improved the specificity of the assay (Laeyendecker *et al.*, 2004). It has been suggested that antibodies may exist in African sera which react to the gG-2 protein on the HSV-2 EIA, either due to genetic reasons or as result of a reaction to other endemic infections in the region (Laeyendecker *et al.*, 2004). Another explanation for potential anomalies could be that African cohort may be infected with a more heterogeneous population of viruses or have a different affinity to HSV-2 as measured by the present assay. Although the role of biological contaminants in affecting the assay's fidelity remains unknown, it may be possible that certain contaminating proteins may be more prevalent in some parts of Africa.

To asses the evolution of HSV type-specific antibodies in persons with newly acquired HSV-2 infection we tested prospective samples from two patients. In the first case, we used the HSV type-specific EIA to trace retrospectively the timing of HSV-2 infection in a mother who had transmitted HSV-2 to her neonate. In this case we demonstrated that no HSV-1 and HSV-2 antibodies were present during

pregnancy and immediately after delivery, whereas seroconversion for HSV-2 antibodies in the absence of HSV-1 antibodies was demonstrated during further follow-up. These findings demonstrated a primary HSV-2 infection acquired during pregnancy, possibly in the last part of pregnancy, and that delivery occurred before the maternal development and possible transplacental transfer of HSV-2 type-specific antibodies. This scenario has shown to carry the highest risk of neonatal morbidity (Brown, 2004).

We reviewed a recent clinical case of a 27 year old woman diagnosed with primary HSV-2 infection with encephalitis (data not shown), admitted to Kings College Hospital (London, UK). Serological testing demonstrated an absence of HSV-2 type-specific antibodies at the onset of symptoms which however became detectable within 4 weeks of onset. The absence of HSV-1 antibodies in this case indicated that HSV-2 infection was primary. These findings were consistent with previous observations indicating that in persons with recently acquired genital herpes, the median time to the development of HSV-2 antibodies from the onset of symptoms is 21-23 days as determined by the HerpeSelect HSV-2 EIA (Ashley-Morrow *et al.*, 2003). One study used the HerpeSelect EIA to determine seroconversion times in 113 patients (413 sera) with recently acquired genital herpes. Of the 113 patients, 31 had primary genital HSV-1, 56 had primary HSV-2 and 26 were HSV-1 antibody positive and newly acquired HSV-2. The median interval from onset of symptoms to seroconversion was 25 days, 21 days and 23 days respectively. In addition, the

HerpeSelect EIA was able to detect the HSV-2 seroconversion rate faster than the western blot assay (Morrow *et al.*, 2003).

We also tested the performance of the HSV-2 antibody assay in a population of GUM attendees with known HSV PCR results from genital swabs. HSV-2 antibodies were detected in all patients with a history of recurrent genital herpes and a HSV-2 positive genital swab. Among patients with first-episode genital herpes and a HSV-2 positive HSV-2 swab, 62% lacked HSV-2 antibodies consistent with a newly acquired infection. Conversely, 38% of first-episodes were in fact established and recurrent HSV-2 infections. These data confirmed that differentiation between primary and recurrent infections based on clinical history alone can be inaccurate and that type-specific serology can improve diagnosis by correctly classifying the infection. One limitation of this study was that antibody testing was anonymised. Hence serological follow-up was not possible to demonstrate seroconversion in patients with a newly acquired HSV-2 infection. Among patients with a history of recurrent genital herpes but with a negative HSV PCR, 37% were HSV-2 antibody positive, confirming that HSV-2 type specific serology can be used to identify patients who lack a definitive diagnosis of genital herpes by HSV detection methods. This finding also demonstrates that although PCR significantly improves HSV detection, the combined use of PCR and serology can provide the most sensitive tool for the diagnosis of patients with recurrent genital disease of unknown aetiology.

With the recent advent of HSV type-specific serological tests, it has become possible to use HSV-type specific serology in sexual health settings. Several applications are currently being evaluated including the management of individuals with features suggestive of genital herpes where culture and/or PCR are repeatedly negative. In this context a positive HSV-2 antibody result is consistent with genital HSV-2 infection and may therefore provide the diagnosis. The detection of HSV-1 antibodies however would not differentiate between a genital and an oropharyngeal HSV infection (Ashley & Wald, 1999). Potential applications include management and risk-reduction counseling of patients with/without partners diagnosed with genital herpes. In addition, determining whether HSV infection is new or established in pregnant women with first-episode genital herpes would provide valuable information on the risk of vertical infection in the neonate (Ashley, 1998). HSV-2 type-specific antibody testing may be proposed as a screening tool to diagnose genital herpes in asymptomatic persons and identify those at risk of transmitting the infection to sexual partners or neonates (Wald, 2002; Kimberlin, 2004). This however remains currently controversial.

4. Chapter 4. The clinical epidemiology of genital herpes in genitourinary clinic attendees

4.1 INTRODUCTION

Genital herpes has traditionally been associated with HSV-2 infection. In recent years however, HSV-1 has increasingly been recognised as a common cause of genital herpes. In several cohorts in Europe and the USA (Smith & Robinson, 2002) HSV-1 has accounted for over 50% of first presentations. Among GUM clinic attendees in England and Scotland, 22-71% of genital herpes cases were due to HSV-1 (Woolley & Kudesia, 1990; Ross *et al.,* 1993; Tayal & Pattman, 1994; Scoular *et al.,* 2002),

It is important to determine the HSV type in genital infections, as knowledge of the infecting HSV type provides additional information for management and counselling. The natural history of genital infection differs significantly according to whether HSV-1 or HSV-2 is implicated. Although the duration of HSV-1 and HSV-2 episodes is similar, infection with HSV-1 is milder with fewer recurrences (Engelberg *et al.,* 2003; Benedetti *et al.,* 1994). The risk of a symptomatic recurrence is 50-57% for HSV-1 and 89% for HSV-2 within the first 12 months after first episode disease. For HSV-1 and HSV-2 infections, the average annual recurrence rates are 1.3 and 4 episodes per year respectively (Corey & Holmes, 1983; Engelberg *et al.,* 2003; Benedetti *et al.,* 1994). Although the rates of HSV recurrences decline over time for both viruses, HSV-1 recurrences decline by 50%

within the second year of infection, whereas an appreciable decline in the recurrence rate is not observed with HSV-2 until 3 to 5 years after the first episode (Wald *et al.*, 2000; Benedetti *et al.*, 1999). Sub-clinical shedding is also less frequent with HSV-1 (Koelle & Wald, 2000). Given the important role that sub-clinical shedding plays in transmission, this observation suggests that transmission may occur less easily than transmission of HSV-2.

In the previous chapter we demonstrated that real-time PCR is a sensitive technique for HSV detection in genital swabs. Using this method, we investigated the epidemiological and clinical features of genital herpes in an ethnically diverse population of GUM attendees.

4.2 METHODS

The cohort included 186 consecutive GUM clinic attendees from Kings College Hospital, London who underwent swabbing for suspected genital herpes. The genital swabs were tested by real-time LightCycler PCR and typed as described in the previous chapter. Statistical analyses were performed using SAS version 8; χ^2 or Fisher's exact tests were used for qualitative variables, Mann-Whitney U tests for quantitative variables.

4.3 RESULTS

4.3.1 Study population

The demographic characteristics of our study population are summarised in Table 4.1. We investigated 186 consecutive individuals presenting to the GUM clinic in Kings College Hospital, London with suspected genital herpes. There were 104 males and 82 females, and the median age was 29 years (range 16-67 years). Sexual orientation was predominantly heterosexual (176/186, 95%). A large proportion of the cohort comprised of persons of black-Caribbean ethnicity (76/185, 41%) followed by white (65/186, 35.5%) and black-African ethnicity (44/186, 22%). Of the 76 patients of black-Caribbean ethnicity, 39/76 (51%) patients immigrated to the UK from Jamaica and 37/76 (49%) were born in the UK. Of the 65 patients of white ethnicity, most were born in the UK (56/65, 86%), and the remaining 9/65 (14%) were born in other European countries (n=6) or America (n=3). Of the 41 patients of black-African ethnicity, 29/41 (71%) had immigrated to the UK from Sub-Saharan Africa and 12/41 (29%) were born in the UK (n=11) or France (n=1). The most common countries of origin in Sub-Saharan Africa were Zimbabwe (7/29), Nigeria (7/29), Ghana (5/29), Zambia (3/29), Uganda (2/29), Gambia (1/29), Eritrea (1/29), Ethiopia (1/29) and Kenya (1/29). Finally, there were three patients from the Indian subcontinent and one from the Middle East.

Table 4.1. Demographic and clinical characteristics of patients with suspected genital herpes stratified by gender

		Men n (%)	Women n (%)	P[1]
Number		104	82	
Country of birth	UK	53 (51)	52 (63)	
	Jamaica	26 (25)	13 (16)	
	Other	25 (24)	17 (21)	0.19
Ethnic group	Black-Caribbean	41 (39)	35 (43)	
	White	35 (34)	30 (37)	
	Black-African	24 (23)	17 (21)	
	Other	4 (4)	0 (-)	0.37
Age (years)	Median	30	29	0.37
	Range	17-67	16-61	
Disease	First-episode	60 (58)	54 (66)	
	Recurrent	44 (42)	28 (34)	0.33
Swab site	Penis	85 (82)	-	
	Perineum	9 (9)	9 (11)	
	Urethra	6 (6)	-	
	Scrotum	4 (4)	-	
	Vulva	-	63 (77)	
	Vagina	-	9 (11)	
	Buttock	-	1 (1)	n/a
Onset	<5 days	43 (41)	51 (62)	
	≥5 days	61 (59)	31 (38)	0.008
Ulceration		72 (69)	62 (76)	0.42
HIV status	Negative	53 (51)	31 (38)	
	Positive	11 (11)	8 (10)	
	Not known	40 (39)	43 (52)	0.15

[1]P-value obtained from χ^2, Fisher's exact or Mann-Whitney U tests.

4.3.2 Clinical features of infection

Lesions most commonly occurred in the penile skin in men (85/104, 82%) and the vulva in women (63/82, 77%). Other lesion sites included the perineum, urethra, and scrotum in men, and the vagina and buttocks in women (Table 4.1). Women were significantly more likely than men to present <5 days of the onset of symptoms (51/82, 62% versus 43/104, 41%; P=0.008) (Table 4.1). There were no other significant differences between men and women, except that the 10 homosexual patients were all men. A clinical diagnosis of first episode disease was made in 114/186 (61%) of patients. The remaining 72/186 (39%) reported a history of recurrent genital herpes. No significant differences were seen between first and recurrent episodes in the time of presentation (P=0.10), the presence of visible ulceration on examination (P= 0.48), and the site of presentation in men (P=0.45) and women (P=0.58) (Table 4.2).

Analysis of demographic and clinical features according to ethnic background showed that all men of homosexual orientation were of white ethnicity (P=0.0002) (Table 4.3). Patients of black-African ethnic origin were older than those from other ethnicities (P=0.03). Patients of white ethnicity (38/65, 59%) were the most likely to present ≥5 days of the onset of symptoms, whereas those of black-Caribbean ethnicity (29/76, 38%) were the least likely (P=0.04) (Table 4.3). In the entire cohort, HIV-1 status was negative in 84/186 (45%) and positive in 19/186 (10%) patients. A large proportion of patients declined HIV testing (83/186, 45%). A large proportion of persons of black-Caribbean origin declined HIV testing compared with other ethnicities (P=0.03) (Table 4.3).

Table 4.2. Comparison of clinical characteristics among patients with suspected genital herpes, stratified by first episode and recurrent disease presentations

Clinical characteristics	First episode	Recurrent episode	P[1]
Site among men[2]:			
Penis	51 (85.0)	34 (77.3)	
Perineum	4 (6.7)	5 (11.4)	
Urethra	2 (3.3)	4 (9.1)	
Scrotum	3 (5.0)	1 (2.3)	0.45
Site in women[2]:			
Vulva	43 (79.6)	20 (71.4)	
Perineum	5 (9.3)	4 (14.3)	
Vagina	6 (11.1)	3 (10.7)	
Buttock	0 (-)	1 (3.6)	0.58
Onset			
<5 days	63 (55.3)	31 (43.1)	
>5 days	51 (44.7)	41 (56.9)	0.10
Ulceration:	80 (70.2)	54 (75.0)	0.48
HSV-2 Ab			
Number tested	42	28	
n (%) positive	16 (38.1)	19 (67.9)	0.03

[1]P-value obtained from Chi-squared tests, Fisher's exact tests or Mann-Whitney U tests, [2]Comparisons of site of swab are performed after combining the three smallest groups in each case. Therefore, in men the comparison is between the penis and other sites, and in women it is between the vulva and other sites.

Table 4.3. Demographic and clinical characteristics of patients presenting with suspected genital herpes, stratified by ethnic group[1]

		Black-Caribbean	White	Black-African	
		n (%)	n (%)	n (%)	P[2]
Number		76	65	41	
Gender	Male	41 (54)	35 (54)	24 (59)	
	Female	35 (46)	30 (46)	17 (42)	0.87
Sexual orientation	Heterosexual	76 (100)	56 (86)	41 (100)	
	Homosexual	0 (-)	9 (14)	0 (-)	0.0002
Age	Median	28	29	33	0.03
	Range	16-57	17-61	18-67	
Disease	First-episode	51 (67)	38 (59)	22 (54)	
	Recurrent	25 (33)	27 (42)	19 (46)	0.32
Swab site	Penis	34 (45)	29 (47)	18 (44)	
	Perineum	6 (8)	7 (11)	5 (12)	
	Urethra	2 (3)	1 (1.5)	3 (7)	
	Scrotum	3 (4)	-	1 (2)	
	Vulva	27 (36)	22 (34)	14 (34)	
	Vagina	4 (5)	5 (8)	-	
	Buttock	-	1 (1.5)	-	n/a
Onset	<5 days	47 (62)	27 (42)	19 (46)	
	≥5 days	29 (38)	38 (59)	22 (54)	0.04
Ulceration		57 (75)	43 (66)	32 (78)	0.34
HIV status	Negative	30 (40	34 (52)	18 (44)	
	Positive	6 (8)	4 (6)	9 (22)	
	Not known	40 (53)	27 (42)	14 (34)	0.03

[1]Four patients with other ethnicities were excluded, [2]P-value obtained from χ^2-squared tests, Fisher's exact tests or Mann-Whitney U tests.

4.3.3 Detection of HSV in genital swabs by real-time PCR

Real-time PCR detected HSV DNA in 108/186 (58%) genital swabs (Table 4.4). The majority of HSV positive patients were infected with HSV-2 (101/108, 93.5%), and only a small proportion with HSV-1 (7/108, 6.5%). Typing results were confirmed as described in chapter 3. The proportion of HSV-1 positive swabs was 4/58 (7%) in men, 3/51 (6%) in women, 5/62 (8%) in first-episode presentations and 2/46 (4%) in recurrent disease.

Statistical analysis by univariable analysis revealed a positive HSV PCR to be associated with heterosexual risk group, <5 days of onset and presence of genital ulceration (Table 4.4). Multivariable analysis confirmed all three factors to be independently associated with a positive HSV PCR after adjustment (Table 4.4). No significant differences were evident in HSV detection rates between men and women (P=0.31), first and recurrent episodes (P=0.26), and HIV status (P=0.18). Analysis by ethnic origin showed that a similar proportion in each group tested positive for HSV (P=0.24). Of those who tested positive for HSV, HSV-1 was significantly associated with white ethnicity, whereas HSV-2 was common in persons of black-African and black-Caribbean ethnicity (P=0.006) (Figure 4.1).

Table 4.4. Characteristics of patients whose genital swabs tested positive for HSV.

		HSV PCR positive[1]		Multivariable Analysis	
		n (%)	P[2]	OR (95% CI)[3]	P
Gender	Male	57 (55)			
	Female	51 (62)	0.31		
Ethnic group	Black-Caribbean	49 (65)			
	White	33 (51)			
	Black-African	25 (61)			
	Other	1 (25)	0.20		
Sexual orientation	Heterosexual	106 (60)			
	Homosexual	2 (20)	0.02	0.16 (0.03-0.83)	0.03
Disease	First-episode	62 (54)			
	Recurrent	46 (64)	0.26		
Onset	<5 days	62 (66)		2.00 (1.04-3.81)	0.04
	≥5 days	46 (50)	0.04		
Ulceration	No	15 (29)			
	Yes	93 (69)	0.0001	5.63 (2.73-11.61)	0.0001
HIV status	Negative	51 (61)			
	Positive	14 (74)			
	Not known	43 (52)	0.18		
Age	Those with negative PCR	29 (16-54)			
	Those with positive PCR	30 (17-67)	0.84		

[1]HSV positive results included both HSV-1 and HSV-2 positive swabs, [2]P-value obtained from χ^2-squared tests, Fisher's exact tests or Mann-Whitney U tests, [3]95% Confidence Interval.

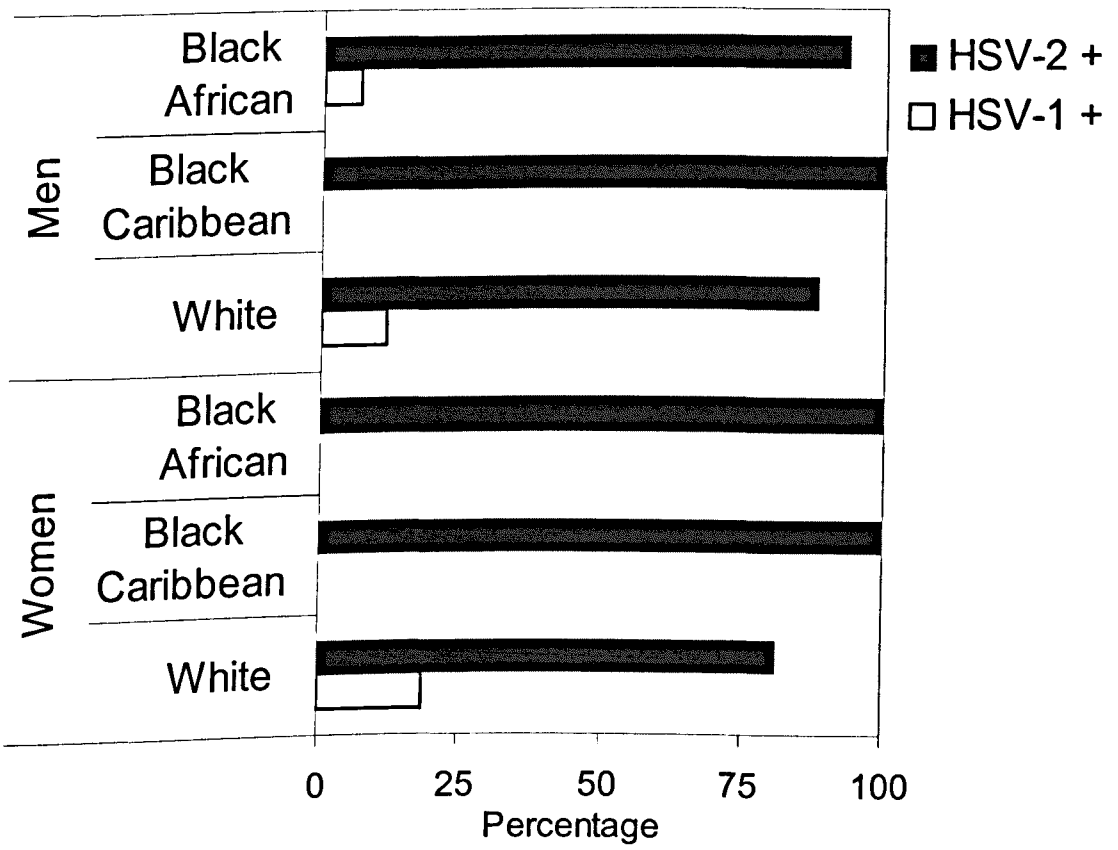


Figure 4.1. Contribution of HSV-1 and HSV-2 to genital herpes, by ethnicity. Among persons whose genital swabs tested HSV positive, HSV-1 was detected more commonly in those who were white, while HSV-2 was more common in those of black-African and black-Caribbean ethnicity (P=0.006).

4.4 DISCUSSION

The results presented in this chapter indicated that epidemiological trends in genital herpes due to HSV-1 or HSV-2 vary between ethnicities in the UK. Ethnicity did not predict the likelihood of a HSV positive genital swab and did not modify the clinical features of the infection; however, it significantly influenced

the type of HSV associated with the infection and the delay between onset of symptoms and presentation. Early studies in UK (Sheffield, Newcastle upon Tyne, and Watford) and Scotland (Edinburgh, Fife and Glasgow) have shown HSV-1 to account for 22-71% of cases of genital herpes, with a higher prevalence among young women with first-episode disease (Woolley & Kudesia, 1990; Ross *et al.*, 2003; Tayal & Pattman, 1994; Slomka *et al.*, 1998; Thompson, 2000; Scoular *et al.*, 2002).

Among GUM attendees in Edinburgh, HSV-1 was more commonly reported as the cause of genital herpes in patients born in the UK than HSV-2 (Ross *et al.*, 1993). The results from this study are in contrast with other studies done in the UK. HSV-2 was the most prevalent cause of genital herpes in our ethnically diverse population. A significant association was found between black ethnicity and HSV-2 infection. To explain this observation, one could propose that childhood acquisition of HSV-1 among those of lower socioeconomic status could render them less susceptible to genital HSV-1 infection during adulthood. One could also speculate that persons of black ethnicity were less likely than patients of white ethnicity to attend the GUM clinic if infected with HSV-1.

HSV-1 oropharyngeal transmission occurs commonly in children, and may influence the risk of genital HSV-1 acquisition in later life. Epidemiological surveys in England and Wales, including a small proportion (2%) of persons from London, indicated that currently HSV-1 acquisition occurs less commonly in children, leaving large numbers of sexually active adults susceptible to genital HSV-1 infection as they lack protective antibodies (Vyse *et al.*, 2000). In contrast,

HSV-1 seropositivity rates remain >91% in children in several African countries (Smith & Robinson, 2002). In the UK and the USA, the seroprevalence of HSV-1 is higher in persons of black ethnicity compared to those of white ethnicity (Gibson *et al.,* 1990; Breinig *et al.,* 1990). In a study of 3533 pregnant women attending an antenatal clinic in London, HSV-1 seroprevalences were 75%, 96% and 92% among black women born in the UK, Africa and the Caribbean respectively, compared to 80% of women born in the UK of white ethnicity (Ades *et al.,* 1989). The population-based AIDS in multiethnic neighbourhoods study among 1770 individuals in the USA, revealed 61% and 46% of persons of black and white race respectively to be HSV-1 seropositive (Siegel *et al.,* 1992).

Another important contributing factor is that the GUM clinic in Kings College Hospital (London, UK) serves a socio-economically disadvantaged population, which may be more likely to acquire oropharyngeal HSV-1 infection in childhood (Cowan *et al.,* 2002; Barlow *et al.,* 1997; Narouz *et al.,* 2003). This may explain the relatively low prevalence of HSV-1 in women of white ethnicity, with 2/9 (22%) first-episode diseases and 1/7 (14%) recurrences due to HSV-1, which is in contrast with the recognised strong association between genital HSV-1 and female gender (Scoular *et al.,* 2002).

An increase in oral sex practices is thought to be a contributory factor to the increasing prevalence of genital HSV-1 infections, resulting from transmission from persons infected with HSV-1 oropharyngeally. There is limited evidence to indicate that oral sex practices vary among patients of different ethnicity. Surveys conducted among GUM attendees in London between 1993-1994, indicated

women of white origin were more likely to have practiced fellatio than women of black-Caribbean ethnicity, and that both fellatio and cunnilingus were practised more frequently by men of white ethnicity than men of black ethnicity (Radcliffe *et al.,* 1993; Evans *et al.,* 1998). One study conducted among students in the USA, revealed that persons of white ethnicity were significantly more likely to engage in oral sex than those of black ethnicity (Johnson *et al.,* 1994). In contrast, another study reported the proportion of black American teenage men who engaged in oral sex to have more than doubled between 1988 - 1995, an increase that brought them in line with levels of oral sex reported by white and Hispanic adolescent males (Gates & Sonenstein, 2000). However, findings from this study must be interpreted with caution as population demographics was lacking. As the prevalence of genital HSV-1 infections increases in the community, genital transmission of HSV-1 also becomes more likely. In this context assortative (like with like) sexual mixing may contribute to maintaining the differences in prevalence of genital HSV-1 among ethnic groups.

Independent predictors of a positive HSV PCR were early presentation after the onset of symptoms, heterosexual risk group and presence of visible genital ulceration on examination. Women were more likely than men to present early after the onset of symptoms. An additional interesting finding was that patients of black-Caribbean ethnicity presented earlier than other groups. A large proportion of patients of black-Caribbean ethnicity also declined HIV testing compared to patients of other ethnicity. HIV infection rates are estimated to be significant in this group, ranging from 0.6-0.7% among heterosexuals to over 10% among male homosexuals (Dougan *et al.,* 2004). Our findings support results from the

Unlinked Anonymous Prevalence Monitoring Programme which showed 50-73% of black-Carribean persons in the UK left the GUM clinic unaware of their HIV infection (Dougan *et al.*, 2004).

In summary, this study highlighted differences in genital HSV-1 and HSV-2 infection trends between different ethnicities in the UK. HSV PCR was used for the virological diagnosis of genital herpes, due to its increased sensitivity, as demonstrated in the previous chapter. The prevalence of HSV-2 infection was high among the cohort, with a higher prevalence in patients of black ethnicity. These findings have important implications for strategies to prevent the transmission of genital herpes.

Chapter 5. Sequence analysis of the HSV-2 UL14 gene in clinical isolates from an ethnically diverse population with genital herpes

5.1 INTRODUCTION

The genome of Herpes simplex virus (HSV) contains at least 74 genes, many of which have unknown function. The UL14 gene is a core gene of herpesviruses. It is conserved among all alpha herpesviruses and homologues are found in beta and gamma herpes viruses. UL14 encodes a 219 amino acid protein with a 32kDa molecular mass, which is a minor component of the viral tegument produced late in the replication cycle (McGeoch *et al.*, 1988; Dolan *et al.*, 1998). The coding region overlaps substantially with that of the UL13 gene.

The UL14 gene is dispensable for HSV replication in cultured cells and is therefore classified as an accessory gene. Nonetheless accessory genes appear to play essential roles in HSV replication and infectivity (Nishiyama, 2004). Consistent with this observation, a mutant with a 4 bp deletion in the central part of UL14 (amino acid position 96-98) showed an extended growth cycle *in vitro* and appeared compromised in efficient transit of virus particles from the infected cell (Cunningham *et al.*, 2000). In mice injected intracranially, the 50% lethal dose of the mutant virus was significantly reduced. In addition, recovery of the mutant virus from the sacral ganglia of mice injected peripherally was significantly reduced relative to that of wild-type virus, suggesting

defective establishment of latency or defective reactivation from latency (Cunningham *et al.*, 2000).

Although the function of the UL14 gene has not yet been fully characterised, HSV-1 UL14 appears to function as a molecular chaperone and is involved in the translocation to the nucleus of the viral proteins VP26 (a minor capsid protein) and UL33 (a DNA cleavage/packaging protein) (Roizman, 1996; Yamauchi *et al.*, 2001). The UL14 protein shares several properties of heat shock proteins (Hsp), including nuclear translocation upon heat shock, ATP deprivation and osmotic shock (Yamauchi *et al.*, 2002a). The protein contains an amino acid sequence that is homologous to part of the substrate-binding domain of the Hsp70 family. In HSV-2, the homologous region stretches a region beginning with Arg60 to Ala74. Two arginine residues in this region (Arg 60 and Arg64) have been found to be important for the translocation to the nucleus of VP26 (Yamauchi *et al.*, 2002a). Further data have suggested that the UL14 protein plays an anti-apoptotic role, which is consistent with the role of other molecular chaperones such as Hsp27 and Hsp70 (Yamauchi *et al.*, 2003).

Although the UL region of the HSV-1 strain 17 has been sequenced (McGeoch *et al.*, 1988; Dolan *et al.*, 1998), there are no data currently on the degree of conservation of the UL14 gene. Most studies on the molecular diversity of HSV-2 have focused on the DNA polymerase (Podzorski *et al.*, 2000; Sun *et al.*, 2003) or the thymidine kinase (TK) gene (Chibo *et al.*, 2004; Nagamine *et al.*, 2000). HSV genetic variability has been used to identify HSV genotypes associated with distinct anatomical sites (Buchman *et al.*, 1978;

Chaney *et al.*, 1983; Umene & Yoshida, 1993; Umene *et al.*, 1996; Umene & Kawana, 2000; Remeijer *et al.*, 2002; Yoshida & Umene, 2003; Roest *et al.*, 2004) and disease patterns (Yoshida & Umene, 2003; Buchman *et al.*, 1978; Chaney *et al.*, 1983; Umene & Yoshida, 1993; Umene & Kawana, 2000; Yoshida & Umene, 2003) and to investigate the molecular epidemiology of HSV infections (Sakaoka *et al.*, 1985; Sakaoka *et al.*, 1987; Umene & Sakaoka, 1991; Sakaoka *et al.*, 1995; Maertzdorf *et al.*, 1999; Sakulwira *et al.*, 2003; Norberg *et al.*, 2004; Bowden *et al.*, 2004). These studies have most commonly employed restriction fragment length polymorphism (RFLP) to investigate HSV variability. Although geographical clustering of HSV-2 strains has been suggested (Sakaoka *et al.*, 1987; Sakaoka *et al.*, 1995), there remains limited information on the variability of HSV-2 sequences.

The aim of this study was to characterise sequence variability of the HSV-2 UL14 gene in clinical isolates obtained from an ethnically diverse cohort of patients with genital herpes.

5.2 METHODS

Genital swabs (Section 2.1.1) from 26 GUM clinic attendees from Kings College Hospital, London who presented with clinical symptoms of genital herpes and had a HSV-2 positive swab by real-time LightCycler PCR (Section 2.5.1.1, Section 2.5.2.1) were processed. The viral DNA was extracted using the QIAamp DNA mini kit (Qiagen, UK) as previously described (Section 2.5.1.1, Section 2.6.1.2). PCR amplification of the 659 bp spanning position from 28229 to 28888 of the HSV-2 UL14 gene was performed

with dNTPs, forward and reverse primers as described earlier (Section 2.6.1.2). For negative controls, DNA specimens were replaced with deionised water. Positive controls included previously known clinical specimens that were typed HSV-2 positive by both virus culture and PCR. Amplicons of 500 bp were identified by ethidium bromide staining and the UL14 protein region was sequenced (spanning amino acids 1-137) using identical forward and reverse primers on an ABI 3100 analyser, under conditions previously described (Section 2.6.2, Section 2.6.3). Sequence analysis was performed using Chromas software version 1.45 and confirmed HSV-2 using BLAST. Sequence alignment was performed using ClustalW software version 1.82. (http://www.ncbi.nlm.nih.gov/BLAST). Only one entry for the HSV-2 UL14 nucleotide sequence was found in the Genbank database (Accession no: CAB06774). Sequence alignment was performed using ClustalW software version 1.82 (http://clustalw.genome.jp). The 26 nucleotide sequences were submitted to GenBank and assigned accession numbers (Table 5.3).

5.3 RESULTS

5.3.1 Study population

The cohort included 26 GUM attendees (13 men and 13 women) from Kings College Hospital, London who were diagnosed with HSV-2 infection. The demographic characteristics of the study population are summarised in Table 5.1. Our cohort was ethnically diverse and included 8/26 (31%) black-African patients of which 5/8 were born

in Nigeria (n=3) or Zimbabwe (n=2) and migrated to the UK in adult life, and 3/8 were born in the UK. Among the 13/26 black-Caribbean patients, 5/13 (39%) were born in Jamaica and migrated to the UK in adult life, and 8/13 (61%) were born in the UK. Among those of white ethnicity, 5/26 (19%), all were born in the UK. Genital lesions were seen most commonly in the penile skin of males (12/13, 92%) and in the vulva of females (9/13, 69%). Other sites of presentation included the perineum and the buttock.

Table 5.1. Demographic characteristics of GUM attendees with genital herpes

Characteristics		n (%)
Gender	Male	13 (50)
	Female	13 (50)
Age (median, range in years)		32 (17-60)
Sexual orientation	Heterosexual	25 (96)
	Homosexual	1 (4)
Ethnicity	Black-Caribbean	13 (35)
	Black-African	8 (31)
	White	5 (19)
Clinical Episode	First	15 (58)
	Recurrent	11 (42)
Onset of symptoms	< 5 days	17 (65)
	≥ 5 days	9 (35)
HIV status	Positive	4 (15)
	Negative	10 (38)
	Unknown	12 (46)

5.3.2 Nucleotide differences of the HSV-2 UL14 gene among individuals diagnosed with genital herpes

HSV-2 UL14 sequences were conserved and no differences were observed in the nucleotide sequences of 18/26 (69%) samples. The 8/26 (31%) individuals with variant nucleotide sequences included 4 white patients all born in the UK, 2 black-African (one born in the UK and one from Zimbabwe) and 2 black-Caribbean persons from Jamaica. There were equal proportions of men and women and 4/8 patients presented with first-episode disease.

Sequence alignment revealed that sequence differences evident in the 8 patients were primarily nucleotide substitutions. These were scattered across 8 different regions of the UL14 gene (Table 5.2) at positions 28237 bp, 28347 bp, 28362 bp, 28469 bp, 28601 bp, 28603 bp, 28606 bp and 28617 bp. One patient showed a single insertion at position 28636 bp.

Among the patients with variant sequences, 7/8 showed a single nucleotide substitution, whereas one 39 year old black-African male patient born in the UK showed a more divergent sequence with 4 nucleotide substitutions. The sequence variation in the 8 patients included two silent or synonymous substitutions and 8 non-synonymous nucleotide substitutions (Table 5.2) that translated into single point mutations at amino acid positions 3, 40, 45, 125, 126 and 130 of the UL14 protein. One black-Caribbean

patient from Jamaica showed a single nucleotide insertion at bp position 28636, which translated into 7 point mutations spanning amino acid positions 129-136. All 26 nucleotide sequences were submitted to the GenBank database (Table 5.3).

Table 5.2. Demographic characteristics and nucleotide sequence differences in patients infected with variant strains of HSV-2

Patient[a]	Country of birth	Ethnic group[b]	Clinical episode	Nucleotide position (bp)	Nucleotide variation[c]			Amino acid change[d]
F 19	UK	W	Recurrent	28237	G → C	SS	NSy	R3P
M 20	UK	W	First	28237	G → C	SS	NSy	R3P
F 21	Zimbabwe	BA	Recurrent	28237	G → C	SS	NSy	R3P
M 22	UK	BA	Recurrent	28347	G → A	SS	NSy	D40N
				28603	C → T	SS	NSy	A125V
				28606	A → C	SS	NSy	N126T
				28617	T → C	SS	NSy	W130R
M 23	UK	W	First	28362	G → T	SS	NSy	G45C
M 24	UK	W	Recurrent	28469	C → A	SS	Sy	-
F 25	Jamaica	BC	First	28601	G → T	SS	Sy	-
F 26	Jamaica	BC	First	28636	+ C	SI	NSy	L129P, W130V, D132G, G133R, D134R, L135P, A136G

[a]M= male; F= female; [b]W= white, BA= black African, BC= black-Caribbean; Nucleotide variation from consensus, whereby the first letter denotes the consensus nucleotide and the second letter denotes the change; SS= single substitution, SI= single insertion; Sy= synonymous NSy= non syno nymous; [d]Amino acid change from consensus sequence, whereby the first letter denotes the amino acid consensus and the second letter denotes the base change

Table 5.3. HSV-2 UL14 amino acid sequences (amino acids 1 to 137) in 26 patients infected with HSV-2.

Position*	1	10	20	30	40	50	60	70	80	90	100	110	120	130	137
Control sequence	MSRDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASLWGDGDLAD														
1-18: DQ224079-DQ224081 DQ231011-DQ231025 24: DQ223892 25: DQ223893	MSRDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASLWGDGDLAD														
F19: DQ222885	MSPDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASLWGDGDLAD														
M20: DQ223889	MSPDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASLWGDGDLAD														
F21: DQ223890	MSPDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASLWGDGDLAD														
M23: DQ223891	MSRDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVCAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASLWGDGDLAD														
M22: DQ222886	MSRDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQNPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAVTASLRGDGDLAD														
F26: DQ223894	MSRDASHAALRRRLAETHLRAEVYRDQTLQLHREGVSTQDPRFVGAFMAAKAAHLELEARLKSRARLEMMRQRATCVKIRVEEQAARRDFLTAHRRYLDPALSERLDAADDRLADQEEQLEEAAANASPVGGRRPGD														

*Point mutations are shaded. The HSV-2 UL14 control sequence was derived from the GenBank database (Accession no: CAB06774). GenBank accession numbers for the 26 nucleotide sequences are denoted alongside patient identifiers; *Amino acid position calculated from start of the 219 amino acid UL14 protein.

5.4 DISCUSSION

In this study we demonstrated a high degree of conservation of the HSV-2 UL14 gene among HSV-2 infected GUM attendees in London. RFLP analysis has demonstrated the presence of genomic polymorphism among strains of the same HSV type (Umene & Yoshida, 1993; Umene & Sakaoka, 1991; Sakulwira *et al.*, 2003). Several epidemiological studies have used this approach to genetically characterise HSV infections, with particular emphasis on HSV-1 (Maertzdorf *et al.*, 1999; Roest *et al.*, 2004; Umene *et al.*, 2003; Yoshida & Umene, 2003). Molecular epidemiological studies demonstrated that HSV-1 RFLP patterns show geographical clustering (Sakaoka *et al.*, 1985). There is limited information available for HSV-2 (Sakaoka *et al.*, 1995; Norberg *et al.*, 2004; Bowden *et al.*, 2004). Early studies on 123 unrelated HSV-2 isolates from Japan and Sweden showed distinct cleavage site distributions (Sakaoka *et al.*, 1987). This finding is consistent with data from a study of 307 HV-2 isolates from four countries (Japan, Korea, Sweden and USA) which similarly showed geographical clustering of HSV-2 strains (Sakaoka *et al.*, 1995).

Association between HSV genotypes and both anatomical site of infection and disease patterns have been proposed. Early studies that analysed HSV-1 DNA variation using RFLP analysis identified 35 HSV-1 genotypes, of which F1 and F35 were predominant (Umene & Yoshida, 1993). The F1 genotype was associated with multiple clinical patterns. In contrast, the F35 genotype showed a characteristic association with eczema herpeticum (Umene *et al.*, 1996). Genotypic variation has also been observed among

sequential isolates of 11/30 (37%) patients with recurrent epithelial keratitis (Remeijer *et al.*, 2002). HSV-1 genotypes can also be specifically isolated from primary or recurrent genital lesions and the HSV-1 F1 genotype has been shown to be closely associated with recurrent infections (Umene & Kawana, 2000).

There are no data available on the sequence variability of the HSV-2 UL14 gene. Our findings demonstrated that the gene sequence if conserved, with nucleotide changes occurring in 8/26 (31%) patients. The substitutions translated into amino acid changes in 6 of the 8 patients. There were no consistent substitution patterns, as changes involved amino acid position 3 in three patients, 45 in one patient, and 40, 125, 126 and 130 in another patient. There was a high degree of conservation of the protein region spanning amino acids 46 to 124. One black Caribbean female showed a single nucleotide insertion which resulted in multiple amino acid changes between positions 129-136. To determine whether the nucleotide insertion and the amino acid changes represented a new allele polymorphism or a result from a typing or sequencing error, patient specimens underwent virus culture, PCR and sequencing. Reproducible results were obtained, suggesting the above were not due to sequencing artefacts. In addition, no trends in HSV variability were observed according to ethnic background or clinical presentation.

Previous data indicate that the amino acid sequences in the N-terminal region (residues 60-74) of HSV-2 UL14 protein sequence shares homologies with the C-terminal region of the substrate recognition domain of Hsp70 (Yamauchi *et al.*, 2002a). Interestingly no changes were observed in the Hsp homologous region containing the two arginine

residues important for the translocation to the nucleus of VP26. A study of BALB/c mice injected intracranially with a HSV-1 mutant containing a 4 bp deletion (spanning amino acid 96-98) demonstrated that 50% lethal dose of the mutant was reduced by more than 30,000-fold and that recovery of the mutant from the latently infected sacral ganglia of mice injected peripherally was significantly less than that of wild-type virus (Cunningham *et al.*, 2000). The region identified as important in attenuating pathogenicity and latency was similarly conserved in the isolates we sequenced. These observations are consistent with the proposed function of UL14 as an Hsp-like molecular chaperone and an important role in virus replication and infectivity.

The observation that a truncated mutant carrying a deletion of 81 amino acids at the N-terminus of the UL14 protein can traffic a fused peptide of at least 13 amino acids intracellularly (Yamauchi *et al.*, 2002b) has led to the speculation that the mutant UL14 may provide a novel strategy for increasing the distribution of physiologically active substances in gene therapy (Nishiyama, 2004). Defining the degree of variation of the protein may help the development of such treatment strategies.

6. Chapter 6. The epidemiology of HSV-1 and HSV-2 infection in HIV-1 infected individuals

6.1 INTRODUCTION

6.1.1 HSV-1 seroprevalence

HSV-1 seroprevalence varies by country and type of population. The National Health and Nutrition Surveys (NHANES II, 1976-80; NHANES III, 1988-94) conducted in the general population in the USA, reported 68% of persons aged 12 years and over to be HSV-1 seropositive (Schillinger *et al.*, 2004). In the UK, HSV-1 seroprevalence in the general population has been shown to be 50% among individuals up to 30 years of age (Vyse *et al.*, 2000) (Table 6.11). A recent cross-sectional survey taken among eight European countries showed the highest age standardised HSV-1 seroprevalence among the general population to be 84% in Bulgaria, 81% in the Czech Republic, 67% in Belgium, 57% in the Netherlands and 52% in Finland (Pebody *et al.*, 2004). The median age of HSV-1 acquisition ranged from 5-9 years in the Czech Republic and Bulgaria to > 25 years in England & Wales, Finland and the Netherlands.

Among high-risk populations, HSV-1 antibody rates may be higher (Table 6.12). Among HIV-1 positive persons attending a GUM clinic in Coventry, 86% of men and 97% of women ($p = 0.14$) were HSV-1 antibody positive (Allan & Das, 2004). Similar rates (94%) have been observed among female commercial sex workers in Mexico (Conde-Glez *et al.*, 1999) and STD clinic attendees (91%) in Uganda (Wagner *et al.*, 1994).

Table 6.1.1. HSV-1 seroprevalence in the general population

Country	Total	Year	%	Reference
France	3192	1994-2002	66	Malvy *et al.*, 2005
England and Wales	5430	1994-1995	24	Vyse *et al.*, 2000
		1986-1987	34	
Germany	3792	1997-1998	83	Hellenbrand *et al.*, 2005
USA	13098	1988-1994	68	Schillinger *et al.*, 2004
Switzerland	3120	1992-1993	80	Bünzli *et al.*, 2004
Rural districts in Japan	614	1973	65	Hashido *et al*, 1999
		1993	70	

Table 6.1.2. HSV-1 seroprevalence in high-risk populations

Country	Setting	Year	n (%)	Reference
Washington, USA	STD clinic attendees with positive genital cultures	1993-1997	1145 (17)	Lafferty *et al.*, 2000
Cape town Johannesburg and Durban, South Africa	STD clinic attendees with GUD	1993-1994	558 (51)	Chen *et al.*, 2000
Ontario, Canada	STD clinic attendees	1994-1995	6555 (56)	Singh *et al.*, 2005
Washington, USA	Lesbian population	1998	392 (46)	Marrazzo *et al.*, 2003
Melbourne, Australia	HIV-1 positive and negative homosexuals in a primary care setting	1999-2000	300 (73)	Russell *et al.*, 2001
Rotterdam, Netherlands	STD clinic attendees	1993 1998	1024 (68) 654 (59)	Roest *et al.*, 2001
Mexico City, Mexico	Female commercial sex workers attending a STD clinic	1992	997 (94)	Conde-Glez *et al.*, 1999

6.1.2 HSV-2 seroprevalence

The prevalence of HSV-2 infection varies among regions and populations. In the USA, a population-based seroprevalence survey conducted in 1990 detected HSV-2 antibodies in 22% of persons aged 12 years and above (Johnson *et al.,* 1989). HSV-2 seroprevalence increased from 16% to 22% between 1978 and 1990 (Janier *et al.,* 1999). In Europe, HSV-2 seroprevalence in the general population ranges between 4% in England and Wales to 24% in Bulgaria (Pebody *et al.,* 2004) (Table 6.2.1). Higher seroprevalence rates have been observed among high-risk populations (Table 6.2.2). In STD attendees, HSV-2 seroprevalence ranges between 22% and 55% in Western Europe (Janier *et al.,* 1999; Roest *et al.,* 2001; Varela *et al.,* 2001; Suligoi *et al.,* 2002), 30% and 64% in the US (Austin *et al.,* 1999; Whittington *et al.,* 2001; Koutsky *et al.,* 1990), and 40% and 65% in Australia (Cunningham *et al.,* 1993; Bassett *et al.,* 1994). In South-East Asia, HSV-2 seroprevalence rates range between 53% and 76% among commercial sex workers (CSW) (Nelson *et al.,* 1997; Limpakarnjanarat *et al.,* 1999).

In Africa, HSV-2 prevalence rates of 40-50% are common in 20 year old persons (Kamya *et al.,* 1995; Greenblatt *et al.,* 1988). In the Mwanza region in Tanzania, a serological survey of a random sample of 259 women and 231 men in 12 rural communities showed that 75% of women $\geq$ 25 years of age and 60% of men $\geq$ 30 years of age were HSV-2 antibody positive (Obasi *et al.,* 1999). However, as with many serological assays, some uncertainty remains about borderline values. The authors reported a risk of overestimation in low prevalence subgroups as the cut-off estimated in the EIA was dependent on the overall prevalence of infection in the population. Similar prevalence rates (58%) have also been observed in a randomly selected population aged 15-54 years

in Uganda (Emonyi *et al.*, 2000). Among high-risk populations, HSV-2 prevalence rates are higher. A multi-centre study involving sex workers in four different African cities reported HSV-2 prevalence rates of 91% in Cotonou, 84% in Yaoundé, 94% in Kisumu and 87% in Ndola (Lagarde *et al.*, 2001).

Table 6.2.1. HSV-2 seroprevalence in the general population

Country	Total (n)	Year	%*	Reference
USA	68000	1976-1994	22	Fleming *et al.*, 1997
England and Wales	5430	1994-1995	3 (M) 5 (F)	Vyse *et al.*, 2000
France	4410	1994-2002	15.5	Malvy *et al.*, 2005
Germany	3792	1997-1998	14	Hellenbrand *et al.*, 2005
Switzerland	3120	1992-1993	19	Bünzli *et al.*, 2004
GuanCaste, Costa Rica	10049	1993-1994	38.5	Rodriguez *et al.*, 2003
Spain	3974	1992-1993	4	Garcia-Corbeira *et al.*, 1999
Masaka, Uganda	294	1989-1990	68	Wagner *et al.*, 1994
Mwanza, Tanzania	490	1993	31	Obasi *et al*, 1999
Rural districts in Japan	614	1973 1993	5.5 6	Hashido *et al*, 1999

*(M) males, (F) females

Table 6.2.2. HSV-2 seroprevalence among high-risk populations

Country	Setting	Year	n (%)	Reference
Baltimore, USA	Intravenous drug users	1999-2002	543 (40)	Plitt *et al.*, 2005
Washington, USA	Lesbian population	1998	392 (8)	Marrazzo *et al.*, 2003
Ontario, Canada	STD clinic attendees	1994-1995	6555 (19%)	Singh *et al.*, 2005
Rotterdam, Netherlands	STD clinic attendees	1993 1998	1024 (30) 654 (22)	Roest *et al.*, 2001
Gijon, Spain	STD clinic attendees	1996-1997	374 (25)	Varela *et al.*, 2001
Northern Italy	STD clinic attendees	1997-1998	919 (25)	Cusini *et al.*, 2000
Coventry, UK	HIV-1 infected GUM attendees	2000-2001	96 (20)	Allan & Das, 2004
Dar-Es-Salaam, Tanzania	STD clinic attendees	1989-1993	294 (43)	Langeland *et al.*, 1998
Pondicherry, India	STD clinic attendees	2001-2003	135 (85)	Shivaswamy *et al.*, 2005
Haifa, Israel	STD clinic attendees	1998-1999	472 (9)	Feldman *et al.*, 2003
Mexico City, Mexico	Female commercial sex workers	1992	747 (65)	Uribe-Salas *et al.*, 1999
Chiang Rai, Thailand	Sex workers	1991-1994	500 (76)	Limpakarnjanarat *et al.*, 1999
Dhaka, Bangladesh	Hotel-based sex workers	2002	400 (34.5)	Nessa *et al.*, 2004

6.1.3 The relationship between HSV-1 and HSV-2 seroprevalence

A decrease in HSV-1 acquisition during childhood is thought to have contributed to increased HSV-2 seroprevalence in the developed world (Kinghorn, 1994), suggesting a possible protective effect of HSV-1 against HSV-2 acquisition. Studies have shown up to 90% of adults in Africa to be infected with HSV-1 (Whitley & Roizman, 2001). Prior HSV-1 infection may therefore provide little or no protection against HSV-2 acquisition, leading to increased numbers of asymptomatic HSV-2 seroconversions (Langenberg *et al.*, 1999) and influence the clinical severity of disease (Corey *et al.*, 1999; Brown *et al.*, 1997). This is consistent with recent findings from vaccine trials which have demonstrated an absence of cross-reactive immune protection (Stanberry, 2004; Aurelian, 2004; Jones & Cunningham, 2004).

The most recently studied subunit vaccine is from GlaxoSmithKline (GSK). Two phase III trials were conducted, one among HSV-1 and HSV-2 seronegative individuals and the second among HSV-2 seronegative persons with or without HSV-1 seronegativity. Both trials demonstrated a significant protective effect (73% efficacy) with reduced clinical symptoms of genital herpes among HSV-1 and HSV-2 seronegative women (Stanberry *et al.*, 2002). The second trial demonstrated no significant effects in HSV-1 seropositive men and women. It has been postulated that previously established immunity might interfere with the vaccination and contribute to the lack of efficacy in HSV-1 seropositive women. An alternative explanation would be that the latter may not enhance levels of protection provided by earlier HSV-1 infection. In either case, a significant effect on the proportion of the disease is unlikely, because approximately 80 to 95% of adults are infected with HSV-1 (Xu *et al.*, 2002; Aurelian, 2004).

6.1.4 The relationship between HSV-2 seroprevalence and HIV-1 infection

In recent years, a strong epidemiological association has emerged between HSV-2 and HIV-1 infection (Severson *et al.*, 1999; Hook *et al.*, 1992; Celum *et al.*, 2004). The strong epidemiological association between HIV-1 and HSV-2 may reflect common risk factors for infection. Previous studies have documented an association between HSV-2 infection and sexual behaviour (Cowan *et al.*, 1994; Johnson *et al.*, 1989). A recent behavioural surveillance survey in 16 states in the USA, between 2000-2002 showed that 36% of HIV-1 positive MSM with non-steady partners did not know the serostatus of their most recent partner, with only a quarter of this population reporting condom use (CDC, 2004). There is also evidence for a more direct interaction between the two viruses. In prospective studies, HSV-2 has shown to increase the risk of HIV-1 acquisition and transmission by approximately two-fold (Blower & Ma, 2004; Celum *et al.*, 2004; Weiss, 2004).

A meta-analysis of 9 cohort and nested case-control studies (Table 6.3) showed that the population-attributable risk percentages for HIV-1 infection were 19% and 47% with a HSV-2 seroprevalence of 22% and 80% respectively (Wald & Link, 2002). Although these studies address heterosexual transmission, additional studies conducted in 47 HIV-1 seroconverting homosexual men and 57 matched controls in San Francisco, USA, found that HSV-2 seropositive men were significantly more likely to acquire HIV-1 than those that were seronegative for HSV-2 (Holmberg *et al.*, 1988). A similar nested case-control study of homosexual men in Amsterdam also documented increased risk of HIV-1 acquisition associated with HSV-2 seropositivity (Keet *et al.*, 1990).

Table 6.3. Meta-analysis of 31 studies to assess risk estimates of HIV-1 infection in HSV-2 infected individuals*

Study type and sub-group	No of studies	Summary estimate**	95% CI
Cohort and nested case-control	9	2.1	1.4 - 3.2
Women	1	0.5	0.2 - 1.1
Heterosexual men	4	2.2	1.3 - 3.8
MSM	4	2.1	1.3 - 3.4
Developing countries	5	2.1	1.0 - 4.2
Developed countries	4	2.1	1.3 - 3.4
Case-control and cross-sectional	22	3.9	3.1 - 5.1
Women	13	3.9	2.7 -5.5
Heterosexual men	9	4.1	2.9 - 5.8
MSM	3	4.3	2.4 - 7.6
Developing countries	15	4.6	3.5 - 5.9
Developed countries	7	2.9	1.7 - 4.7

*Adapted from Wald & link, 2002; **Summary estimates for cohort and nested case-control studies, were calculated using relative risk and for case-control and cross-sectional studies, odd ratios were used.

Extensive observational studies leave little doubt that STDs facilitate HIV transmission through direct biological mechanisms. Genital ulceration due to HSV-2 is thought to increase HIV-1 transmission by increasing the levels of HIV-1 shedding through genital lesions, and by providing a portal entry into the host (Mbopi-Keou *et al.*, 2003). These findings have been supported by a study conducted in Rakai, Uganda, which indicated an increased risk of HIV-1 transmission in persons with genital ulcer disease (Gray *et al.*, 2001). In addition, there is supporting evidence from studies that show HSV-2 and HIV-1 to interact at a molecular level, and up-regulate HIV-1 replication through the long-terminal repeat region (Golden *et al.*, 1992; Mosca *et al.*, 1987). In a transient co-infection system, HSV immediate early regulatory proteins (ICP0, ICP4 but not ICP27) have shown to transactivate HIV-1 LTR, although the ability of these proteins to produce this effect depends on the cell-line used (Ostrove *et al.*, 1987). Transfected ICP0 was shown to activate HIV-1 LTR expression from an integrated chimeric HIV-1 provirus (Chapman *et al.*, 1991). In addition, interactions between ICP0 and RNA binding protein Tat has shown to stimulate HIV-1 LTR-driven transcription (Schafer *et al.*, 1996).

Activated CD4 cells recruited by inflammatory responses to HSV-2 to the site of herpetic lesions may provide an ideal target for HIV-1. These data indicate plausible biological interactions between the two viruses and suggest that genital herpes plays an important role in the dynamics of HIV-1 infection. The objectives of the study were to determine the rates of HSV-1 and HSV-2 seroprevalence in persons newly diagnosed with HIV-1 infection and the associated risk factors. In addition, we wished to determine the role of HSV-2 seroconversion following HIV-1 diagnosis. Among HSV-2 seropositive persons, we determined the clinical expression of genital herpes.

6.2 METHODS

6.2.1 Study cohort

Our cohort comprised of 850 newly diagnosed HIV-1 seropositive persons who attended the Caldecot Centre, King's College Hospital, between January 1986 and December 2001, and were followed up for at least one year after HIV-1 diagnosis. HSV-type specific serology was performed on this study population using stored serum samples collected at the time of HIV-1 diagnosis (Section 2.1.2, Section 2.4.1, Section 2.4.3, Section 2.4.4). Of those individuals who initially tested as being HSV-2 seronegative, a subset of 200 persons was randomly selected. Persons with at least one year of follow-up were retained, leaving 123 patients who underwent HSV-2 serological testing at ≥1 year after HIV-1 diagnosis.

A review of patient's clinical and laboratory records was performed to collect demographic data, determine the occurrence of a clinical diagnosis of genital herpes. Information on a virological diagnosis of genital herpes by virus culture, and diagnosis of other STDs was also collected. When assigning risk group status, bisexual males were categorised under the homosexual risk group. Ethnicity was assigned according to UK Census categories. The clinical diagnosis of genital herpes was made by the observation of genital lesions suggestive of genital herpes and a history of symptoms consistent with the diagnosis. Virus culture was the routine diagnostic test used for virological confirmation. The diagnosis of syphilis was based on dark ground microscopy of ulcer exudate and serological testing by *Treponema pallidum* EIA IgG and IgM, quantitative RPR, and fluorescent antibody absorbed test. Testing for other STDs included serological

assays for the diagnosis of hepatitis A and hepatitis B; culture, enzyme immunoassay (EIA), and molecular testing of urine and urethral, cervical and anal swabs for the diagnosis of Chlamydia infection; microscopic examination and culture of urethral, cervical, anal and throat swabs for the diagnosis of gonorrhoea, and microscopy and culture of vaginal swabs for the detection of *Trichomonas vaginalis*. The diagnosis of human papilloma virus (HPV) infection was based on either the clinical observation of genital warts or the detection of HPV-associated changes on cervical or anal cytology. The diagnosis of scabies was made clinically.

6.2.2 HSV type-specific serology

HSV-1 and HSV-2 serology was performed using the HerpeSelect IgG EIA (Focus Technologies, Cypress, California, USA). Specificity was increased by raising the assay cut-off for a positive result to 3.1 as described in chapter 3b and by repeat testing of sera from Sub-Saharan Africa by inhibition EIA.

6.2.3 Statistical Analysis

Univariable comparisons were performed using χ^2 or Fisher's exact tests for qualitative variables and Mann-Whitney U tests for quantitative variables. Factors independently associated with i) the presence of HSV-2 antibodies, ii) a clinical diagnosis of genital herpes among those with HSV-2 antibodies, and iii) a positive HSV-2 swab in those with a clinical diagnosis of genital herpes were identified by multivariable logistic regression analysis. All statistical analyses were performed using Statistical Analysis System (SAS) version 8.

6.3 RESULTS

6.3.1 Patient demographics

Between 1986 and 2001, 850 individuals (median age 33 years, range 17-71 years) were newly diagnosed with HIV-1 infection. Of these, 534 (63%) were men (Table 6.4). Risk groups for HIV-1 infection were heterosexual contact in 467 (55%), homosexual contact in 338 (40%) homosexuals, and less commonly other risk groups including intravenous drug use in 39 (4%). The cohort was ethnically diverse (Table 6.4). Among patients of black-African ethnicity, 351/385 (91%) were born in sub-Saharan Africa (95 Uganda, 65 Zimbabwe, 33 Zambia, 27 Nigeria, 23 Ivory Coast, 19 Ghana, 17 Kenya, 11 Ethiopia, 10 Sierra Leone, 9 Tanzania, 7 Zaire, 6 Burundi, 6 Rwanda, 4 Malawi, 3 Somalia, 3 Cameroon, 3 Congo, 2 Gambia, 2 Guinea, 2 Togo, 2 Eritrea, 1 Angola and 1 Benin), 24/385 (6%) in the UK and 10/385 (3%) in other countries (6 South Africa, 3 France and 1 USA). Among 83/850 (10%) patients of black-Caribbean ethnicity, 44/83 (53%) were born in the UK, 32/83 (39%) were born in Jamaica, and 7/83 (8%) were born in other areas of the Carribean Islands. Among patients of white ethnicity, 263/371 (71%) were born in the UK, 82/371 (22%) in other areas within Europe (21 Italy, 15 Spain, 11 Ireland, 10 Portugal, 10 France, 3 Germany, 3 Greece, 3 Cyprus, 2 Albania, 1 Belgium, 1 Norway, 1 Poland and 1 Finland) 10/371 (3%) in the USA/Australia, and 16/371 (4%) born in other countries (7 Brazil, 2 Colombia, 2 South Africa, 1 St. Lucia, 1 Zimbabwe, 1 China, 1 Russia and 1 Singapore) . Finally, there were 11 patients from other ethnicities (1 from South America, 1 from Asia, 2 from the Middle East and 7 from the Indian subcontinent).

Table 6.4. Demographic characteristics and predictors of HSV-2 serostatus in 850 persons diagnosed HIV-1 positive between 1986 and 2001.

Characteristics		Number (% of total population)	No. HSV-2 seropositive (%)[d]	P[a]
Gender	Male	534 (63)	298 (56)	0.0001
	Female	316 (37)	239 (76)	
Risk group	Heterosexual	467 (55)	351 (75)	0.0001
	Homosexual	338 (40)	167 (49)	
	IVDU[b]	39 (4)	17 (44)	
	Other[c]	6 (1)	2 (33)	
Ethnicity	Black-African	385 (45)	300 (78)	0.0001
	White	371 (44)	181 (49)	
	Black-Caribbean	83 (10)	53 (64)	
	Other	11 (1)	3 (27)	
HSV-1 serostatus	Positive	752 (88)	479 (64)	0.45
	Negative	98 (12)	58 (59)	

[a]P value determined by univariable analyses; [b]IVDU: intravenous drug users; [c]Other risk group: exposed to contaminated blood or blood products; [d]% value relates to the number HSV-2 seropositive and not to the total population tested.

6.3.2 HSV-1 seroprevalence at the time of HIV-1 diagnosis

At the time of HIV-1 diagnosis, 752 (88.5%) patients were seropositive for HSV-1. HSV-1 seropositivity was 289/752 (38%) among women and 463/752 (62%) among men (data

not shown). Seroprevalence was 369/372 (99%) in persons of black-African ethnicity, 307/307 (100%) in persons of white ethnicity, and 45/67 (67%) in persons of black-Caribbean ethnicity (data not shown).

6.3.3 HSV-2 seroprevalence at the time of HIV-1 diagnosis

HSV-2 specific antibodies were detected in 537 (63%) of patients. Univariable analyses indicated that HSV-2 antibody positivity at the time of HIV-1 diagnosis was associated with female gender (P=0.0001), heterosexual risk group (P=0.0001), black-African or black-Caribbean ethnicity (P=0.0001) and older age (P=0.01) (Table 6.5). As most heterosexual persons were women, a possible confounding association between gender and risk group could not be excluded. To investigate further, two separate multivariable models were used for the two variables. In both models, a positive antibody result was strongly associated with black ethnicity and older age at the time of HIV-1 infection (Table 6.6). After adjustment for these variables, female gender (P=0.03; OR 1.51; 95% CI [1.03-2.20]) and heterosexual risk group (P=0.04; OR 1.62; 95% CI [1.02-2.57]) were independently associated with a positive HSV-2 antibody result (Table 6.6). There was no association with HSV-1 serostatus.

Table 6.5. HSV-2 seroprevalence among 850 HIV-1 positive persons, stratified by age.

Women				Men			
Age	No	No. HSV-2 positive (%)		Age	No	No. HSV-2 positive (%)	
All ages (17-66)	316	239	(76)	All ages (17-71)	534	298	(56)
17-21	14	8	(57)	17-21	11	5	(45)
22-26	65	49	(75)	22-26	42	13	(31)
27-31	87	68	(78)	27-31	120	57	(48)
32-36	74	51	(69)	32-36	162	95	(59)
37-41	38	31	(82)	37-41	109	72	(66)
42-46	23	19	(83)	42-46	52	28	(54)
>46	15	13	(87)	>46	38	28	(74)

Table 6.6. Predictive factors for HSV-2 seropositivity at the time of HIV-1 diagnosis, identified by multivariable logistic regression analyses.

	Odds ratio	95% CI[a]	P
Model 1: Including Gender			
Female	1.51	1.03-2.20	0.03
White/other	1	NA[b]	NA[b]
Black-African	3.25	2.25-4.71	0.0001
Black-Caribbean	1.84	1.12-3.04	0.02
Age (per 5 years older)	1.18	1.08-1.30	0.0004
Model 2: Including Risk group			
Heterosexual	1.62	1.02-2.57	0.04
White/other	1	NA[b]	NA[b]
Black-African	2.70	1.64-4.43	0.0001
Black-Caribbean	1.66	0.98-2.81	0.06
Age (per 5 years older)	1.16	1.06-1.27	0.001

[a]CI: Confidence Interval; [b]NA not applicable

6.3.4 HSV-2 seroincidence after HIV-1 diagnosis

Following the diagnosis of HIV-1 infection, 123 patients who were HSV-2 antibody negative at the time of HIV-1 diagnosis and who remained in follow-up for ≥ 1 year were tested at subsequent time points for the development of HSV-2 specific antibodies. The median HSV-2 EIA index value among the 123 HSV-2 seronegative patients was 0.28 (range 0-0.87). Persons in the prospective cohort had a median age of 32 years (range 17-70) at the time of HIV-1 diagnosis, which was made in 1990-2000 (median 1997). Over median 5 years follow-up (range 2-12 years) 12/123 patients (10%) seroconverted for HSV-2 antibodies. The median index value of the 12 patients before and after HSV-2 seroconversion was 0.25 (range 0.08-0.74) and 4.54 (range 3.52-15.98) respectively. The cohort included 98/123 (80%) males, 80/123 (65%) homosexuals, 43/123 (35%) heterosexuals, and 87/123 (71%) white, 25/123 (20%) black-African and 11/123 (9%) black-Caribbean patients. The incidence rate was 1.8 cases/100 person-years (95% CI: 0.8-2.8). Seroconversion occurred at a median of 4 years (range 2-11 years) after HIV-1 diagnosis.

Among the same 123 patients, 101 (82%) were tested for other STDs at a median of 2 time-points (range 1-12) over median 5 years, according to standard clinical protocols. Overall, 54/123 (44%) received a diagnosis of one (n=38), two (n=15) or three (n=1) STDs. The diagnosed infections included HPV (45/123, 37%), gonorrhoea (13/123, 11%), chlamydia (6/123, 5%), acute syphilis (3/123, 2%), and other infections (acute hepatitis B, n=2; acute hepatitis A, n=1; scabies, n=1) (Table 6.7). The diagnosis of HPV infection was based on the detection of genital warts in 39/45 (87%) patients and the detection of cytological abnormalities of cervical or anal smears in the absence of visible

genital warts in 6/45 (13%) patients. A significant association was found between HSV-2 seroconversion after HIV-1 diagnosis and a diagnosis of HPV infection (P=0.005), gonorrhoea (P=0.05) or other infections (acute hepatitis A, acute hepatitis B or scabies, P=0.0001). Other predictors of HSV-2 seroconversion could not be identified due to small numbers. Those who seroconverted included 11/98 (11%) males and 1/25 (4%) females, 7/80 (9%) homosexuals and 5/43 (12%) heterosexuals, and 9/87 (10%) white and 3/25 (12%) black-African patients.

Table 6.7. Occurrence of sexually transmitted infections and HSV-2 seroconversion during median 5 years of clinical follow-up among 123 HIV-1 positive persons, according to gender and sexual orientation.

Population		HPV[a]	Gonorrhoea	Chlamydia	Acute syphilis	Other[b]
Total		45	13	6	3	4
Gender	Males	37	13	2	2	4
	Females	8	0	4	1	0
Risk group	Homosexuals	31	13	1	1	3
	Heterosexuals	14	0	5	2	1
HSV-2 seroconversion		10	4	0	0	4

[a]HPV: Human papilloma virus infection, including genital warts and HPV-related cytological abnormalities in cervical or anal smears; [b]Other: Hepatitis B (n=2), Hepatitis A (n=1), Scabies (n=1).

6.3.5 Clinical and virological diagnosis of genital herpes among HSV-2 seropositive persons

A clinical diagnosis of genital herpes was made in 116/549 (21%) HSV-2 seropositive patients. None of the 12 HSV-2 seroconverters received a diagnosis of genital herpes. Predictive factors for a clinical diagnosis of genital herpes in univariable analyses were male gender, non heterosexual risk group and white ethnicity (Table 6.8). The year of HIV-1 diagnosis was significantly different in HSV-2 seropositive patients who received a clinical diagnosis of genital herpes (median 1997, range 1990-2001) compared to those who were clinically undiagnosed (median 2000, range 1986-2001) (P=0.0001). Among individuals diagnosed HIV-1 positive before 1997, 63/140 (45%) received a clinical diagnosis of genital herpes compared with 42/397 (11%) patients diagnosed 1997 onwards (P=0.0001). No association was found between the clinical diagnosis of genital herpes and HSV-1 serostatus (Table 6.8). The median age at HIV-1 diagnosis was 33 years (range 18-70) in HSV-2 seropositive patients with a clinical diagnosis of genital herpes and 34 years (range 17-71) in those without a clinical diagnosis (P=0.79). Multivariable logistic regression analysis confirmed the strong correlation between pre-1997 HIV-1 diagnosis and a clinical diagnosis of genital herpes. The adjusted odds ratio (OR) was 5.11 (95% Confidence Interval, CI 3.28-7.98; P=0.0001). In addition, in multivariable analysis there remained a significant association between heterosexual risk group and a reduced likelihood of receiving a clinical diagnosis of genital herpes: OR 0.55; 95% CI 0.35-0.86; P=0.0001.

Of the 116 persons who were clinically diagnosed with genital herpes, 46 (40%) genital swabs yielded an HSV-2 positive result by virus culture (Table 6.8). In univariable

analyses, the year of HIV-1 diagnosis was the only significant predictor of a culture positive swab. The median year of HIV-1 diagnosis was 1996 (range 1990-2001) in patients with a positive swab and 1999 (range 1991-2001) in patients without a virological diagnosis by virus culture (P=0.0006). Those diagnosed prior to 1997 were four times more likely to have a positive HSV-2 swab than those diagnosed subsequently (OR 4.01; 95% CI 1.78-9.02; P=0.0008). Median age at HIV-1 diagnosis was 31.5 years (range 18-70) in patients with a positive swab and 34 years (range 20-53) in those without a HSV positive culture result (P=0.10). There was no significant difference in the yield of a positive genital swab between heterosexuals and other risk groups (Table 6.8).

Table 6.8. Predictive factors for a clinical and virological diagnosis of genital herpes among HSV-2 seropositive individuals, identified by univariable analyses

Factors		No. with clinical diagnosis of genital herpes (%)[a]	P	No. with HSV-2 positive swab by virus culture (%)[b]	P
Gender	Male	76 (25)	0.02	26 (34)	0.15
	Female	40 (17)		20 (50)	
Risk group	Homosexual	50 (30)	0.005	16 (32)	0.33
	Heterosexual	61 (17)		28 (46)	
	Other	5 (26)		2 (50)	
Ethnicity	White	54 (30)	0.003	18 (33)	0.43
	Black-African	55 (18)		25 (45)	
	Other	7 (12)		3 (43)	
HSV-1 serostatus	Positive	101 (21)	0.51	41 (41)	0.80
	Negative	15 (26)		5 (33)	

[a]Among those who were HSV-2 seropositive; [b]among those with a clinical diagnosis of genital herpes.

6.4 DISCUSSION

HSV-2 seroprevalence was 63% among patients who received a diagnosis of HIV-1 infection between 1986 and 2001, confirming the strong epidemiological association between the two infections. The prevalence was significantly higher than that observed in the general UK population. In adults in the general population in England and Wales, the prevalence of HSV-2 antibodies is 4% in those aged >12 years (Pebody *et al.*, 2004) and 10% among those aged >16 years (Morris-Cunnington *et al.*, 2004). The absolute HSV-2 seroprevalence figures presented in the latter study should be interpreted with care, since the Focus EIA test has shown to produce false positive results in African sera (Chapter 3b). In many sub-Saharan African countries HSV-2 seropositivity rates are 40-50% or higher in the general population (Kamya *et al.*, 1995; Greenblatt *et al.*, 1988). In a random sample of individuals aged 15-54 years in Uganda, HSV-2 seropositivity was 58% (Emonyi *et al.*, 2000). In rural communities in the Mwanza region of Tanzania, a random sample showed that 75% of women above 25 years of age and 60% of men above 30 years of age had HSV-2 antibodies (Obasi *et al.*, 1999).

Earlier studies among STD clinic attendees in London have shown HSV-2 prevalence to range between 23 and 26% (Pebody *et al.*, 2004; Evans *et al.*, 2003). However, HSV-2 prevalence data among HIV-1 positive persons in the UK are very limited. In one study, among 92 persons, HSV-2 seroprevalence was 50% among men and 94% among women and the rates of infection were higher in persons of black ethnicity than white ethnicity (Allan & Das, 2004).

Consistent with previous findings (Wald, 2004; Malkin, 2004; Weiss, 2004, Fleming *et al.,* 1997) HSV-2 seropositivity increased with increasing age and was associated with female gender. Previous evidence has suggested HSV-2 seroprevalence to increase with age, and plateau at about 40 years in high prevalence regions such as sub-Saharan Africa (Smith & Robinson, 2002). Age-related risk factors are likely to reflect the cumulative number of sexual partners, age of initiation of sexual activity, and duration of sexual activity (Wald, 2004; Cowan *et al.,* 1994). However, no information on sexual behaviour was available in this study. The high levels of HSV-2 seroprevalence in our cohort are consistent with findings of several previously reported studies. The population based AIDS in Multiethnic Neighbourhoods (AMEN) study, found highest HSV-2 seropositive rates in African-American women than Caucasian women and men (Siegel *et al.,* 1992). The association with female gender is believed to reflect both biological and behavioural factors, including exposure to infection of a large mucosal area in vaginal intercourse and sexual contact with older partners who are more likely to be HSV-2 seropositive (Wald, 2004).

Among HIV-1 infected individuals, HSV-2 seropositivity was also associated with black ethnicity. This finding is in line with previous studies in HIV-1 negative cohorts, indicating that HSV-2 infection rates are generally higher in persons of black ethnicity than in those of white ethnicity (Wald, 2004, Fleming *et al.,* 1997; Evans *et al.,* 2003; Narouz *et al.,* 2003). However, these studies may be limited by population size. In our cohort, a large proportion of persons of black ethnicity originated from sub-Saharan Africa and migrated to the UK in adult life. A smaller proportion of persons were of Caribbean origin, approximately half of whom emigrated from Jamaica to the UK in adult life. In African HIV-1 positive populations, HSV-2 seroprevalence remains high

(Wald, 2002; Freedman & Mindel, 2004; Gwanzura *et al.,* 1998; Mbopi-Keou *et al.,* 2000; Weiss *et al.,* 2001) with rates >80% reported in selected cohorts in Zimbabwe (Gwanzura *et al.,* 1998), Malawi (Sutcliffe *et al.,* 2002), the Central African Republic (Wald, 2004), and Cameroon (Eis-Hubinger *et al.,* 2002) (Table 6.9). Data from the Caribbean are limited, but in one study 77% of 107 HIV-1 positive adults in Barbados were HSV-2 seropositive (Levett, 2005). These data must be interpreted with care due to low population numbers. An increase in the number of participants may have led to more significant findings. The high rates of HSV-2 seropositivity found among persons of black-African and black-Caribbean ethnicity were in agreement with high HSV-2 seroprevalences found in the respective countries of origin. Consistent with this observation, HSV-2 seroprevalence was significantly higher among persons born outside of the UK than in those born in the UK (data not shown).

Table 6.9. The seroprevalence of HSV-2 infection among HIV-1 positive individuals worldwide.

(Town), Country	Year	Gender/Setting	Median age (range)	Total (%HSV-2+)	Reference
(Bangui), Central African Republic	1998-1999	Women	27 (15-48)	58 (91)	Mbopi-Keou *et al.*, 2000
(Cape Town, Durban, Johannesburg), South Africa	1993-1994	Men/GUD	26 (15-65)	196 (63)	Chen *et al.*, 2000
(Greater Harare), Zimbabwe	Pre-1997	Male/factory workers	(≤25->45)	191 (83)	Gwanzura *et al.*, 1998
(Cité Soleil), Haiti	Pre-1992	Women	26 (15-40)	95 (88)	Boulos *et al.*, 1992
(Coventry), UK	2000-2001	Men and women	35 (21-80)	96 (67)	Allan & Das, 2004
(Seattle), Washington	1989-1995	Women	27	60 (75)	Hitti *et al.*, 1997
(Seattle), Washington	1983-1986	Homosexuals	28	100 (66)	Koutsky *et al.*, 1990

Heterosexual risk group was a strong predictor of HSV-2 seropositivity, although due to the relatively small number of heterosexual males a possible confounding association with gender could not be excluded. The relationship between HSV-2 seroprevalence and sexual orientation is not well established. In HIV-1 negative cohorts, no difference in seropositivity was found according to gender or sexual orientation (Cusini *et al.*, 2000). This might be explained by study limitations including reduced population size, and younger age of women at enrolment with reduced sexual exposure. Furthermore, discrepancies which exist between comparisons must take into account differences between serological tests. In a cross sectional study of 380 HIV-1 positive individuals in Rome, Italy, 33% were HSV-2 seropositive, with higher rates in men (38%) than in women (17%) and in homosexuals (55%) than heterosexuals and IVDUs (15%) (Suligoi *et al.*, 2002). The higher prevalence among men and homosexuals can be accounted by differences in the study population tested. These findings are in conflict with the large body of data indicating a higher prevalence of HSV-2 infection in women than in men and illustrate that the predictors of HSV-2 serostatus are numerous and diverse.

Studies in Europe have shown lower HSV-2 infection rates than those documented in sub-Saharan Africa. Studies among European HIV-1 positive cohorts have shown HSV-2 seroprevalence to be 40-54% in men who were either homosexuals (Bystricka *et al.*, 2000) or predominantly homosexuals (Wutzler *et al.*, 2000; Rabenau *et al.*, 2002) and 42-66% in women (presumably predominantly heterosexuals) (Wutzler *et al.*, 2000; Rabenau *et al.*, 2002; Van Benthem *et al.*, 2001). Among HIV-1 positive persons in the USA, HSV-2 antibodies were reported in 63% of heterosexual males (Hook *et al.*, 1992), 66% of homosexual males (Stamm *et al.*, 1988) and 75-78% of heterosexual women (Hook *et al.*, 1992; Hitti *et al.*, 1997). These data suggest an association with female gender rather

than sexual orientation, although a direct comparison of a large number of homosexual and heterosexual males is lacking.

The EIA assay used to determine HSV-2 serostatus shows a sensitivity of 96% and a specificity of 97% among sexually active adults relative to Western blot. The assay performance is not affected by HIV-1 status (Laeyendecker *et al.*, 2001). The threshold to calculate positive results was increased from 1.1 to 3.1 in order to improve specificity, as discussed in chapter 3. The HerpeSelect EIA has shown problems of specificity with samples from patients from Uganda and Kenya (Hogrefe *et al.*, 2002; Laeyendecker *et al.*, 2001). To address these problems, all samples with equivocal results and 38% of those with positive results by EIA were retested using an Immunoblot assay with 97% sensitivity and 98% specificity. Furthermore positive samples from patients from Uganda or Kenya were retested by inhibition-EIA. Despite these precautions, the absolute HSV-2 seroprevalence figures should be interpreted with caution, because false-positive and false-negative results may have occurred, leading to a 2-3% error in the estimation of the HSV-2 seroprevalence.

The prevalence of HSV-1 infection was high in the cohort. There is conflicting evidence concerning the association between HSV-1 and HSV-2 serostatus, with some studies indicating a protective effect of HSV-1 antibodies against the acquisition of HSV-2 infection. The findings are in agreement with other studies (Cowan *et al.*, 1994; Pebody *et al.*, 2004; Wutzler *et al.*, 2000; Suligoi *et al.*, 2002; Brown *et al.*, 1997; Langenberg *et al.*, 1999) where no association between HSV-2 and HSV-1 serostatus was demonstrated. Furthermore, in HSV-2 seropositive persons there was no association between HSV-1 serostatus and the likelihood of receiving a diagnosis of genital herpes.

Genital herpes was under-diagnosed in the cohort, with only 22% of patients with HSV-2 antibodies receiving a clinical diagnosis and an even smaller proportion having a virus positive swab. A similar finding has been described in other HIV-1 positive cohorts, with a history of genital herpes reported in 25% of HSV-2 seropositive women (Van Benthem *et al.*, 2001) and 33% of homosexual males (Russell *et al.*, 2001). As it occurs in HIV-1 negative persons, the under-diagnosis may reflect the fact that HSV-2 clinical recurrence rates tend to decline with time, or that atypical clinical presentations escape recognition by patients or physicians (Wald, 2004). As shown in previous chapters, suboptimal sensitivity of virus culture may play a significant role, explaining the lack of a virological diagnosis in a large number of HSV-2 seropositive persons with clinical symptoms of genital herpes. Furthermore, the introduction of highly active antiretroviral therapy appeared to have a significant impact on the likelihood of developing symptoms of genital herpes. HSV-2 seropositive patients diagnosed from 1997 onwards were at least four times less likely to receive a clinical diagnosis of genital herpes than patients diagnosed earlier. This is consistent with data showing that HIV-1 positive persons on highly active anti-retroviral therapy reported significantly fewer days with HSV lesions than untreated subjects (Posavad *et al.*, 2004) and that CD4 counts correlate with the clinical expression of genital herpes (Van Benthem *et al.*, 2001). However, due to the unavailability of CD4 counts for the HIV-1 infected cohort, no correlation between HSV-2 seropositivity and CD4 counts could be made.

HIV-1 infected persons remained at risk of acquiring HSV-2 after being diagnosed HIV-1 positive and this risk was associated with the acquisition of other STDs. A diagnosis of HPV infection, gonorrhoea, hepatitis A or B and scabies were strongly associated with

HSV-2 acquisition. Thus HSV-2 seroconversion after HIV-1 diagnosis can be considered as a strong marker of high risk sexual behaviour. A recent study in the UK has shown inconsistent condom use among 300 GUM attendees (Handy, 2004). This is consistent with other studies in Africa, which has shown occasional condom use in only 14% of men and 17% of women (Maharaj & Cleland, 2004). High risk taking behaviour has also been observed among HIV-1 infected homosexual men in the USA. Among 1918 HIV-1 infected homosexual men across 4 cities in the USA, 59% reported having multiple partners, 16% engaged in unprotected anal sexual intercourse with partners who were either HIV-negative or whose serostatus was unknown, and an estimated 30 new infections (80% as a result of sexual interactions with MSM) would be expected among the sex partners of study participants during the 3-month reporting period (The National Institute of Mental Health Healthy Living Project Team, 2004).

These findings indicate that persons diagnosed with HIV-1 continue to be at risk of acquiring HSV-2 despite regular counselling on risk-reduction. There was a high incidence of genital warts and HPV-associated cytological abnormalities in cervical and anal smears among those who seroconverted for HSV-2 antibodies. In addition HSV-2 seroconversion was significantly associated with cases of acute gonorrhoea, acute hepatitis A or hepatitis B, and scabies, all but one occurring among homosexual males. These findings indicate that HSV-2 seroconversion and HPV infection in HIV-1 infected persons may be used as a surrogate marker for high risk sexual behaviour. With evidence for a bi-directional interaction between HIV-1 and other STDs, there remains a need for HIV-1 prevention strategies, education and counselling.

7. Chapter 7. T-cell mediated immune responses to HSV-2 infection in HIV-1 positive individuals

7.1 INTRODUCTION

There is overwhelming evidence that T-cell mediated immunity is important in the control of HSV infection. The vast majority of investigations evaluated the protective role of T-cell mediated immunity in mouse models (Parr & Parr, 1998; Parr & Parr, 2003; Svensson *et al.*, 2005). Early data had suggested that the majority of T-cell activity against HSV resides within the CD4 cell subset, although HSV-specific CD8 T-cells has also been detected (Koelle *et al.*, 2000a; Koelle *et al.*, 2000b; Koelle *et al.*, 1994). More recent studies have provided evidence that both CD4 and CD8 T-cell subsets play an important role in the host defence against HSV infection (Koelle *et al.*, 2005; Posavad *et al.*, 2003; Barcy *et al.*, 2005). In particular, the ability of both CD4 and CD8 T-cells to secrete IFN- γ upon activation has been proposed as a common protective mechanism against the infection (Ellerman-Eriksen, 2005). This is supported by the observation that *in vivo* reactivation of latent HSV can be promoted by anti-IFN-γ treatment (Liu *et al.*, 2001; Liu *et al.*, 2000).

In patients with genital herpes lesions, mononuclear cell infiltrates composed of CD4 and CD8 T cells and macrophages have been demonstrated (Eriksson *et al.*, 2004; Cunnigham *et al.*, 1985; Aurelian *et al.*, 2003). CD4 cells are the first cells to infiltrate the site of infection, followed by CD8 cells 48 hours later (Cunningham *et al.*, 1985). HSV is a

strong inducer of IFN-α (Gobl *et al.,* 1988; Ankel *et al.,* 1998; Honda *et al.,* 2005) and the high concentrations of IFN- γ detected within herpetic lesions (Koelle *et al.,* 1998; Mikloska & Cunningham, 2001) are thought to induce up-regulation of MHC class I and II on infected cells, thereby promoting T-cell recognition (Cunningham & Mikloska, 2001).

Within the ganglia where HSV latency is established, the control of infection appears to be dependent on the infiltration of CD8 T cells and expression of T-cell derived cytokines (Branco & Fraser, 2005; Simmons & Tscharke, 1992). The persistence of CD8 T cells and IFN-γ in the ganglia suggests persistent HSV antigenic expression as a driver of antigen-specific T cells. In support of this hypothesis, limited transcription of immediate early (IE) and early (E) genes, in particular, the ICP4 and thymidine kinase has been documented (Shimeld *et al.,* 1999; Kramer & Coen, 1995). Antigen presentation is thought to occur either through neurons expressing MHC class I, or through satellite cells expressing both MHC class I and II (Nash, 2000). Early studies involving mutant mice lacking CD8 T cells demonstrated impaired control of lytic virus infection and loss of neurons from sensory ganglia (Guidotti *et al.,* 1996). These observations imply a role for CD8 T cells in containing actively replicating virus. One study examining the effect of CD8 cells on ganglionic cultures revealed that ganglionic CD8 T cells also effectively inhibited HSV reactivation during latent infection (Liu *et al.,* 1996). More recently, evidence suggested that IFN-γ can independently arrest HSV-1 reactivation in latently infected neurons by inhibiting ICP0 and gC promoter activity (Decman *et al.,* 2005).

These findings imply that resident CD8 cells and/or lymphocyte-derived cytokines are important in both initial control and suppression of reactivation.

Viral immune evasion strategies are important for establishment and maintenance of infections. HSV can evade immune functions in many ways. Dendritic cells (DC) expressing HSV receptors in the skin and the dermis can be productively infected with HSV (Salio *et al.,* 1999) depending on the maturation status of the DC (Kruse *et al.,* 2000; Mikloska *et al.,* 2001). There is documented evidence that the maturation, expression of co-stimulatory molecules, and antigen presenting capacity of DC can be reduced by HSV (Kruse *et al.,* 2000; Salio *et al.,* 1999).

The virion host shutoff (vhs) protein, a component of the tegument has been shown to be released into the cytosol of infected cells and mediate the rapid shutoff of protein synthesis via degradation of both cellular and viral mRNAs (Everly *et al.,* 2002). Vhs can down-regulate MHC class I and class II molecules (HillT *et al.,* 1994; Tigges *et al.,* 1996; Trgovcich *et al.,* 2002) thereby avoiding T cell recognition of HSV-infected cells (Tigges *et al.,* 1996).

HSV can also inhibit antigen processing and presentation in class I pathways by interaction of the US12 gene product, ICP47, with the transporter associated with antigen processing (TAP) (Hill *et al.,* 1995; York *et al.,* 1994). After primary penetration, HSV protein ICP47 binds to TAP and blocks peptide binding and peptide translocation by TAP. This therefore avoids the loading of peptide epitopes into the binding cleft of MHC-

I molecules. Alternatively, HSV can evade immune attack by inhibiting activities mediated by complement (C) components C3, C5, and properdin (P). HSV can also interfere with activities mediated by the Fc domain of immunoglobulin G (IgG) antibodies. HSV-1 gE can bind to the IgG Fc domain and interfere with C1q binding and antibody-dependent cellular cytotoxicity (ADCC). HSV-1 gC can also bind complement component C3 and its activation products, C3b, iC3b, and C3c, and accelerate the decay of the alternative complement pathway (Fries *et al.*, 1986; Kostavasili, *et al.*, 1997). The HSV non-essential glycoproteins gE and gI can also together form a hetero-oligomer complex that functions as a Fc receptor for IgG. Bipolar bridging of the IgG antibody leads to the disruption of effector functions mediated by the Fc region, including binding of C1q which consequently inhibits the classical pathway for complement activation by blocking opsonization (Nagashunmugam, *et al.*, 1998).

Contrastingly, there is convincing immunological data implicating T-cells in HSV epidermal damage (Aurelian *et al.*, 2003). Erythema multiforme (EM) refers to a broad morphological spectrum of lesions, of which a subset is caused by HSV (HAEM). Histopathological features include accumulation of mononuclear cells, endothelial swelling, and epidermal damage. Prospective studies have shown that the preponderance of cells infiltrating the dermis is primarily $CD4^+$ T cells (Malmstrom *et al.*, 1990; Paquet & Pierard, 1997; Auquier-Durant *et al.*, 2002; Kokuba *et al.*, 1998). Studies have demonstrated the presence of IFN-γ in HAEM to be potential effector of tissue damage (Staats & Lausch, 1993). IFN-γ has shown not to be expressed in healed tissues that are negative for HSV proteins and T-cells, confirming the strong correlation between IFN-γ

presence and HSV-protein expression in skin tissues ($p<0.0001$) (Kokuba *et al.*, 1998). The up-regulation of HLA class I and class II antigen expression by IFN-γ may enhance antigen presentation of keratinocytes (Basham *et al.*, 1984), thereby contributing to the immune-inflammatory process. Supporting this interpretation, virus-induced inflammatory responses can trigger autoreactive T cell generation (Rouse, 1996) and molecular mimicry or release of cellular antigens from lysed cells that express HSV proteins may lead to HAEM pathogenesis (Gyotoku *et al.*, 2002; Koelle *et al.*, 2002).

Among individuals with depressed T-cell function, including HIV-infected persons, the immune mechanisms controlling HSV infection are not well understood. Previous data indicated that proliferative responses are reduced in HIV-positive persons compared to uninfected individuals (Hersh *et al.*, 1985; Wainberg *et al.*, 1987). HIV-positive persons also show lower levels of HSV-specific CD8 T-cells (Posavad *et al.*, 1997). A correlation has been observed between absolute CD4 cell count and memory proliferative responses to HSV (Posavad *et al.*, 1997). Suppression of HIV-1 replication by HAART is paralleled with an increase in CD4 counts (Posavad *et al.*, 2004). In addition, we demonstrated that HIV infected patients who were HSV-2 were four times less likely to receive a clinical diagnosis of genital herpes if diagnosed HIV-1 positive after 1997 (Ramaswamy *et al.*, 2006). This suggests that HAART may play a role in immune recovery. This is further supported by findings that have shown that HAART treated individuals have significantly fewer days of HSV lesions compared to untreated individuals (Posavad *et al.*, 2004). This observation suggests a restoration of HSV immune control.

The aim of this study was to investigate prospectively HSV T-cell immunity in HSV-seropositive HIV-1 infected patients starting HAART. To this end, T-cell IFN-γ recall responses to antigen stimulation were characterised *in vitro* using the ELISPOT assay. Further characterisation was done using intracellular cytokine staining.

7.2 METHODS

7.2.1 Study subjects and sampling

Our cohort comprised 64 HIV-1 positive persons and 20 HIV-1 negative healthy volunteers attending the Royal Free Hospital, London. Control samples from the healthy donors were obtained at 3 time points (weeks 0, 4 and 9) and included 11/20 (55%) persons who were HSV IgG seropositive (positive control group) and 9/20 (45%) persons who were HSV IgG seronegative (negative control group). The Royal Free Hospital HIV-1 positive cohort included four groups of patients: (1) patients starting HAART (prospective cohort); (2) long term slow and non-progressors (LTNP/LTSP) with long-standing HIV-1 infection who had remained disease free and maintained a CD4 cell count above 400 cells/ mm^3 over at least 10 years of follow-up since HIV diagnosis (n=3, sampled at three time points); (3) patients with recently diagnosed HIV-1 infection who were not receiving HAART (n=9, sampled once); and (4) patients on HAART (n=33, sampled once). In addition 10 HIV-infected HSV IgG seronegative patients served as a further negative control group. Blood samples were collected in preservative-free sodium heparin tubes for PBMC isolation (Section 2.1.3). In addition serum samples were

collected for the determination of HSV IgG serostatus by EIA using the HSV 1 and 2 DiaSorin kit (DiaSorin, Berkshire, UK) (Section 2.1.2).

7.2.2 Isolation of PBMC, ELISPOT and flow cytometry

PBMCs were isolated as previously described (Section 2.7.1). For the ELISPOT assay (Section 2.7.2), PBMCs (3×10^5 cells/well) were stimulated in triplicates with HSV-1 and HSV-2 lysates at three different concentrations (1 μg/ml, 5 μg/ml and 10 μg/ml). PHA (5 μg/ml) and tissue culture medium were used as positive and negative controls respectively. Frequencies of antigen-specific IFN-γ secreting cells were calculated as mean of three wells, based on the numbers of responder cells and the number of spots per well after subtraction of background. Spots were enumerated using a Nikon stereomicroscope and an automated ELISPOT reader (Karl Zeiss – Imaging Associates, Germany). Results were recorded as spot forming cells (SFC) per 10^6 PBMC. Since HSV-1 and HSV-2 antigens at 10 μg/ml yielded the highest response, this concentration was used to address HSV-specific IFN- γ responses.

Intracellular IFN-γ production following PBMC stimulation with HSV-1 and HSV-2 lysates (1 μg/ml) was assessed in 2 healthy subjects (1 HSV seropositive, 1 HSV seronegative) using flow cytometry as previously described (Section 2.7.3). Positive and negative controls included staphylococcal enterotoxin B (1 μg/ml) (Sigma, Dorset, UK) and uninfected Vero cell extracts respectively. Cells were stained with the following monoclonal antibodies: IFN-γFITC/CD69-PE/CD4-PerCP/CD3-APC (BD Biosciences,

Oxford, UK), and analysed by a FACScan flow cytometer (Becton Dickinson, Oxford, UK). CD69 is expressed on activated T cells, B cells and NK cells and is one of the cell surface proteins which are rapidly up-regulated after lymphocyte activation. Results were calculated by setting a gate around the $CD3^{+}CD4^{+}$ cell population on a forward versus side scatter dot plot; 10,000 events were collected for each sample. Dead cells and debris was excluded by forward and side scatter gating.

7.2.3 Statistical analysis

Sample size calculations were not carried out as this was a pilot study that investigated the effects of HIV and HSV status on the production of SFCn. In addition, we were not aware of any previous work that would have enabled us to estimate the level of response that may be expected amongst our control population. Furthermore, practical constraints determined that 64 HIV positive individuals and 20 HIV negative individuals were recruited.

7.3 RESULTS

7.3.1 HSV-specific T-cell responses of healthy donors

A total of 20 HIV-1 negative healthy adult volunteers were enrolled in the study, with a median age of 53.5 years (range 29-78 years); 11/20 (55%) were HSV IgG seropositive and 9/20 (45%) were HSV IgG seronegative. The IFN-γ responses to HSV-1 and HSV-2

obtained at 3 time points over 9 weeks are summarised in Table 7.1. At all time points, the mean number of SFC was higher among HSV IgG seropositive patients (>150 SFCs/10^6 PBMC) than among those who were HSV IgG seronegative (<30 SFCs/10^6 PBMC) (Table 7.1). Response to the negative medium control and the positive PHA control were comparable in patients who were HSV IgG seropositive and HSV IgG seronegative. Mean SFC for the negative medium control was 3 ± 3/10^6 PBMC. Mean SFC for the positive PHA control was 542 ± 154/10^6 PBMC. Patient 7 who was HSV IgG seropositive had the strongest PHA and HSV-specific response (821 SFC/10^6 PBMC and 443 SFC/10^6 PBMC respectively).

There were modest variations in the mean number of SFC measured at the three time points and responses to HSV-1 and HSV-2 antigens were similar. Overall, the mean SFC number to HSV-1 and HSV-2 combined among HSV IgG seronegative patients were significantly lower than HSV IgG seropositive patients (28 ± 11 versus 318 ± 90). In the HSV IgG positive donors, responses correlated well with the level of antigenic PBMC stimulation *in vitro*. Figure 7.1 illustrates the IFN-γ response to HSV-1 and HSV-2 antigens of one healthy donor at three different antigen concentrations (1, 5 and 10 μg/ml). The mean number of SFC increased progressively with increasing HSV concentration used for PBMC stimulation.

7.3.2 HSV-specific T-cell responses of HSV IgG seronegative HIV infected patients

Ten HIV-infected patients who lacked serum HSV IgG served as an additional negative control group. Their median CD4 count was 457 cells/mm^3 (range 264-970) (Table 7.2). The mean IFN-γ responses to both HSV-1 (25 SFC/10^6 PBMC) and HSV-2 (27 SFC/10^6 PBMC) were similar to those of HSV seronegative healthy controls (HSV-1, 29 SFC/10^6 PBMC; HSV-2, 27 SFC/10^6 PBMC).

Table 7.1a. IFN-γ responses to HSV-1 and HSV-2 in HSV IgG seronegative healthy donors at 3 subsequent time points (week 0, 4 and 9)

	Mean number of IFN-γ SFC at 3 time points							
	HSV-1				HSV-2			
Patient	Week 0	Week 4	Week 9	Mean	Week 0	Week 4	Week 9	Mean
1	30	40	47	39 ± 8	23	30	47	33 ± 12
3	33	27	20	27 ± 7	33	20	27	27 ± 7
4	30	23	37	30 ± 7	50	33	47	43 ± 9
5	17	10	23	17 ± 7	33	20	37	30 ± 9
6	40	33	27	33 ± 7	33	43	30	36 ± 7
9	37	43	40	40 ± 3	30	27	33	30 ± 3
14	17	10	13	13 ± 3	13	10	7	10 ± 3
17	20	23	27	23 ± 3	23	13	13	17 ± 6
19	37	33	33	34 ± 2	13	17	30	20 ± 9
Mean of group	29 ± 9	27 ± 12	30 ± 10	29 ± 9	28 ± 11	24 ± 11	30 ± 13	27 ± 3

Table 7.1b. IFN-γ responses to HSV-1 and HSV-2 in HSV IgG seropositive healthy donors at 3 subsequent time points (week 0, 4 and 9)

Patient	Age (years)	Mean number of IFN-γ SFCs at 3 time points							
		HSV-1				HSV-2			
		Week 0	Week 4	Week 9	Mean	Week 0	Week 4	Week 9	Mean
HSV IgG seropositive individuals									
2	35	337	300	277	305 ± 30	257	277	287	273 ± 15
7	37	483	460	417	453 ± 34	387	443	470	433 ± 43
8	39	133	170	123	142 ± 25	223	237	260	240 ± 19
10	40	367	323	317	336 ± 27	390	387	353	377 ± 20
11	39	163	173	200	179 ± 19	273	290	277	280 ± 9
12	31	377	337	323	346 ± 28	353	333	313	333 ± 20
13	33	130	157	133	140 ± 15	410	447	430	429 ± 18
15	34	333	323	313	323 ± 10	270	310	320	300 ± 26
16	32	320	313	330	321 ± 9	317	330	353	333 ± 19
18	27	230	260	253	248 ± 16	423	437	450	437 ± 13
20	32	377	387	350	371 ± 19	357	403	430	397 ± 37
Mean of group	34 ± 4	295 ± 115	291 ± 90	276 ± 91	287 ± 95	333 ± 68	354 ± 73	358 ± 75	348 ± 70

Table 7.2. IFN-γ responses to HSV-1 and HSV-2 in HIV-infected HSV IgG seronegative patients

Patient	CD4 counts (cells/mm^3)	Mean number of IFN-γ SFCs	
		HSV-1	HSV-2
29	970	47	30
41	590	37	20
33	470	23	23
47	403	33	30
10	264	3	17
35	395	10	20
61	353	13	20
62	464	23	30
63	530	30	37
64	450	33	43
Mean of group	489 ± 192	25 ± 13	27 ± 9

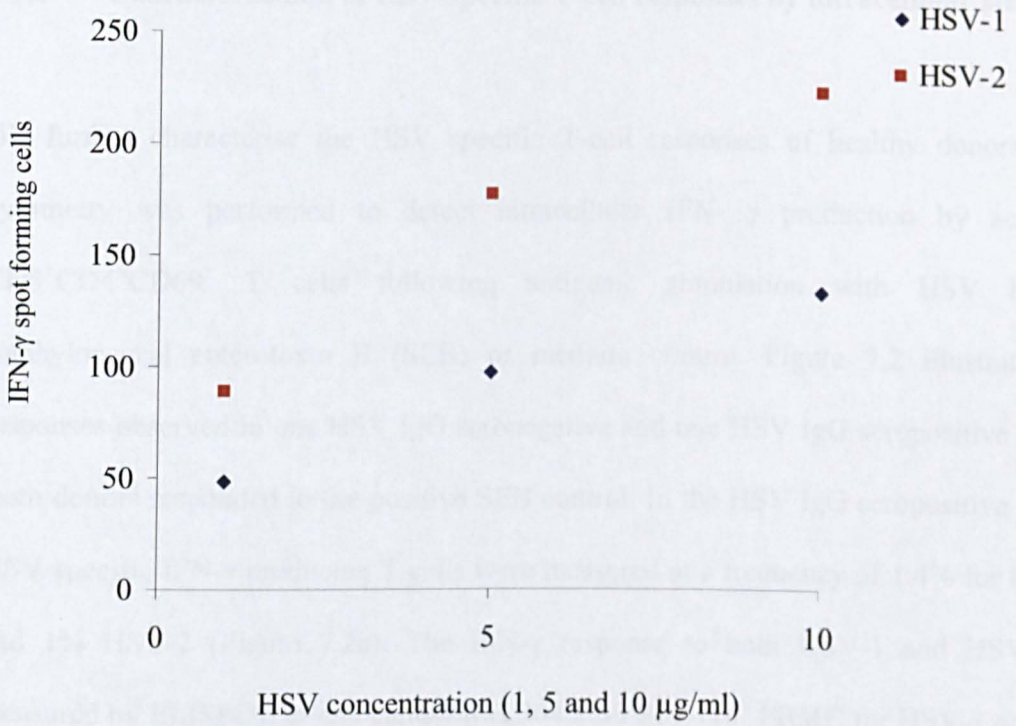
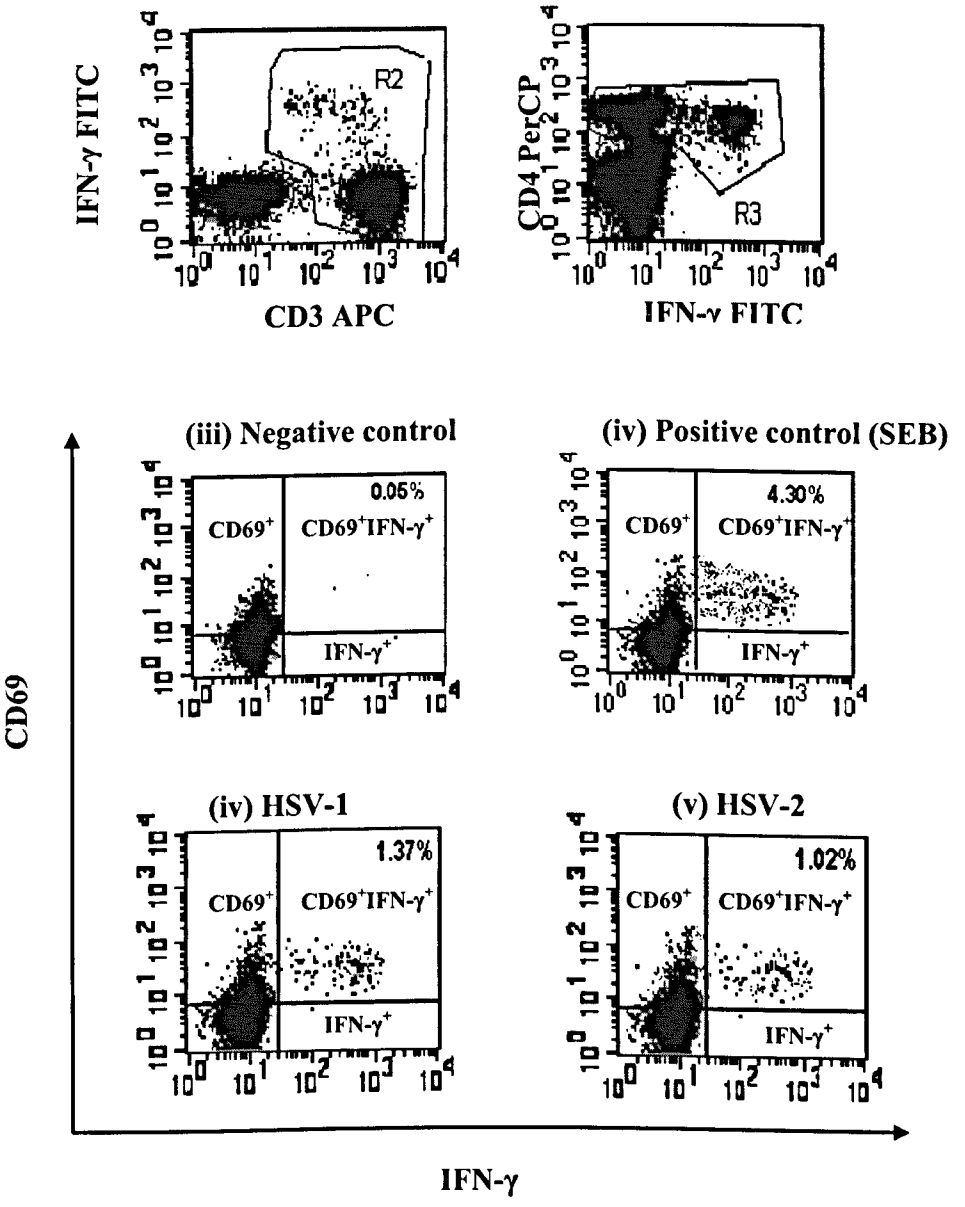


Figure 7.1. Mean (triplicate wells) IFN-γ responses to HSV-1 and HSV-2 at 3 different antigen concentrations (1, 5, and 10 µg/ml) in a HSV IgG seropositive healthy donor

7.3.3 Characterisation of HSV-specific T-cell responses by intracellular staining

To further characterise the HSV specific T-cell responses of healthy donors, flow cytometry was performed to detect intracellular IFN- γ production by activated $CD3^+CD4^+CD69^+$ T cells following antigenic stimulation with HSV lysates, staphylococcal enterotoxin B (SEB) or medium control. Figure 7.2 illustrates the responses observed in one HSV IgG seronegative and one HSV IgG seropositive donor. Both donors responded to the positive SEB control. In the HSV IgG seropositive donor, HSV-specific IFN-γ producing T-cells were measured at a frequency of 1.4% for HSV-1 and 1% HSV-2 (Figure 7.2a). The IFN-γ response to both HSV-1 and HSV-2 as measured by ELISPOT in this patient was 304 ± 30 SFC /10^6 PBMC for HSV-1 and 273 ± 15 SFC/10^6 PBMC for HSV-2. In the HSV IgG seronegative donor (Figure 7.2b), the frequency of IFN-γ producing T-cells after stimulation with HSV antigens was negligible and comparable to the response observed after stimulation with the medium control.

a) IFN-γ responses in a HSV IgG seropositive donor



b) IFN-γ responses in a HSV IgG seronegative donor

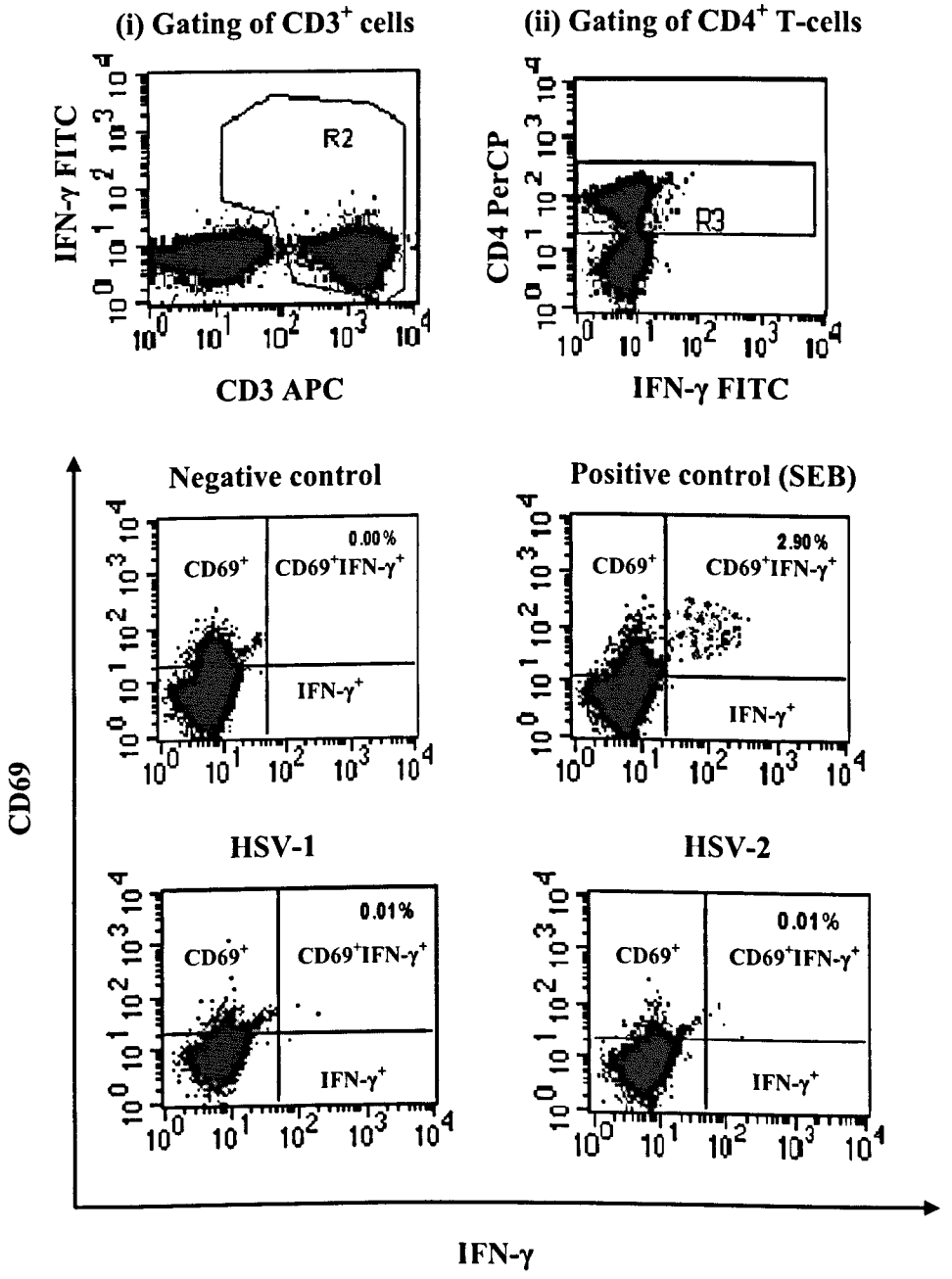


Figure 7.2: Flow cytometric analysis of intracellular IFN-γ expression in a) HSV IgG seropositive individual and b) HSV IgG seronegative individual. PBMCs were stimulated with HSV-1 lysate, HSV-2 lysate, SEB positive control, or uninfected Vero

cell extracts, in the presence of Brefeldin A and anti-CD28 antibody. Cells were permeabilised and stained with anti-CD3-APC, anti-IFN-γ FITC, anti-CD4PerCP and anti-CD69PE antibodies. In the analysis, gating identified lymphocytes (R1, not shown), $CD3^+$ T-cells (R2) and $CD4^+$ T-cells (R3). The IFN-γ expression of activated $CD3^+CD4^+$ T-cells was determined using CD69 as an early T-cell activation marker.

7.3.4 HSV-specific T-cell responses of HIV-1 positive persons

The study cohort included 54 HSV IgG seropositive HIV-1 infected patients. The composition of the study group, patient's age, absolute and percent CD4 cell counts and HIV plasma RNA load as determined at the time of enrolment are summarized in Table 7.3.

Table 7.3. Characteristics of HSV IgG seropositive HIV-1 infected patients

	HIV-1 infected patient groups (n=54)[a]			
	Group 1	Group 2	Group 3	Group 4
Total	9	3	9	33
Time points sampled	Median 5 (range 3-6)	3	1	1
Median age (range) yr	41 (30-45)	42 (28-45)	35 (27-56)	40 (18-71)
Median CD4 (range) cell/mm^3 [b]	208 (5-308)	1219 (480-1261)	453 (320-843)	360 (90-1040)
Median % CD4 cells	18 (1-32)	39 (23-47)	25 (16-40)	20 (7-43)
Median Viral load (log_{10} copies/ml)	4.7	<1.7	4.6	<1.7

[a]Group1: Prospective cohort starting HAART; Group 2: Long-term slow/non-progressors; Group 3: Newly diagnosed patients not on HAART; Group 4: Patients on stable HAART. [b]Laboratory reference CD4 count 400-1500 cells/mm^3.

7.3.4.1 Long term slow and non-progressors (LTNP/LTSP)

Three patients classified as LTNP/LTSP were investigated. The patients (patient 1, 14, 21) had been diagnosed HIV positive 10, 22 and 19 years previously respectively and had shown CD4 cell counts above 400 cells/mm^3, undetectable HIV viral load (<50 copies/ml) and no evidence of clinical progression in the absence of antiretroviral therapy. The three patients had no clinical history of HSV disease and were not on acyclovir. They showed overall HSV-specific T-cell responses similar to those observed

in healthy donors (Table 7.4). Mean responses to HSV-1 and HSV-2 were similar. IFN-γ responses to HSV-1 and HSV-2 antigens were lower in the patient with the lowest CD4 cell counts. Figure 7.3 illustrates an example of IFN-γ responses to HSV-1 and HSV-2 antigens in a patient with CD4 count of 1219 cells/mm^3.

Table 7.4. IFN-γ responses to HSV-1 and HSV-2 in HAART naïve HIV-infected long-term slow and non-progressors

Patient	CD4 counts (cells/mm^3)	Time point (weeks)	Mean number of IFN-γ SFC		HIV plasma viral load (log_{10} copies/ml)
			HSV-1	HSV-2	
1	1219 (47%)	0	427	367	<1.7
	953 (21%)	22	377	333	<1.7
	855 (42%)	43	367	327	<1.7
Mean of 3 time points			390 ± 32	342 ± 21	
14	1261 (39%)	0	437	370	<1.7
	1452 (44%)	16	460	403	<1.7
	1042 (42%)	33	400	353	<1.7
Mean of 3 time points			432 ± 30	376 ± 25	
21	480 (23%)	0	270	290	<1.7
	400 (27%)	6	230	223	<1.7
	370 (28%)	30	193	217	<1.7
Mean of 3 time points			231 ± 38	243 ± 41	

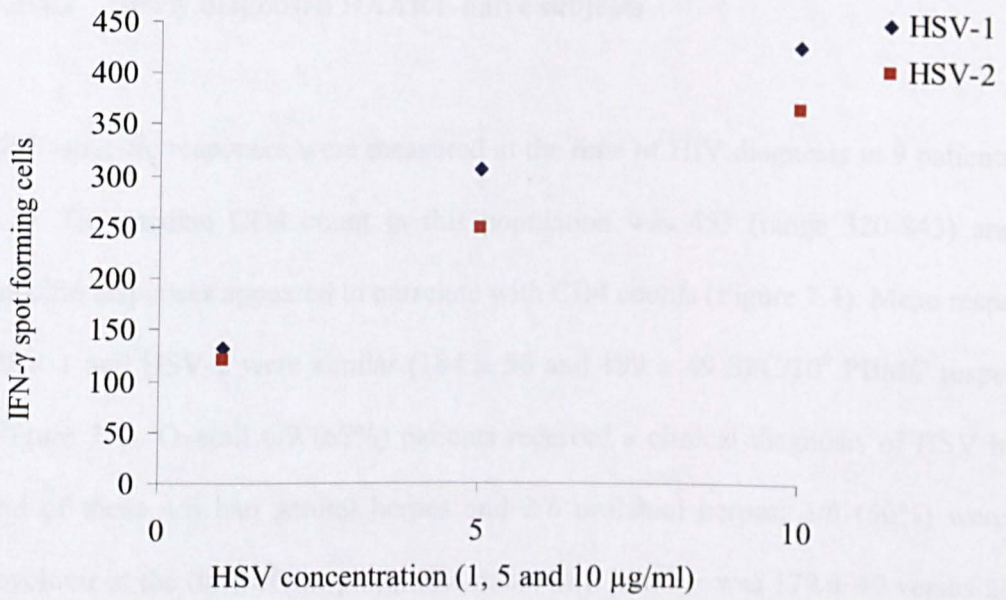


Figure 7.3. IFN-γ responses to HSV-1 and HSV-2 in a LTNP

The plot illustrates IFN-γ responses to three different concentrations of HSV-1 and HSV-2 antigens. At the time of sampling, the patient had been infected for 10 years, was asymptomatic and had a CD4 count of 1219 cells/mm^3 without receiving antiretroviral therapy. The patient did not have a clinical history of HSV disease.

7.3.4.2 Newly diagnosed HAART-naive subjects

HSV-specific responses were measured at the time of HIV diagnosis in 9 patients (Table 7.5). The median CD4 count in this population was 453 (range 320-843) and HSV-specific responses appeared to correlate with CD4 counts (Figure 7.4). Mean responses to HSV-1 and HSV-2 were similar (184 ± 56 and 189 ± 49 SFC/10^6 PBMC respectively) (Figure 7.4). Overall 6/9 (67%) patients received a clinical diagnosis of HSV infection and of these 4/6 had genital herpes and 2/6 orolabial herpes; 3/6 (50%) were taking acyclovir at the time of sampling. The mean SFC number was 179 ± 49 versus 202 ± 56 SFC/10^6 PBMC in persons with or without clinical HSV disease, and 167 ± 54 versus 196 ± 49 SFC/10^6 PBMC in person receiving or not receiving acyclovir.

Table 7.5. IFN-γ responses to HSV-1 and HSV-2 in newly diagnosed HAART-naïve HIV-infected patients

Patient	CD4 counts (cells/mm3)	HIV plasma viral load (log_{10} copies/ml)	Mean number of IFN-γ SFC		History of HSV disease	On anti-HSV therapy at time of sampling
			HSV-1	HSV-2		
44	320 (36%)	4.8	163	170	Oral herpes	No
4	369 (25%)	5.0	110	123	Genital herpes	Yes
11	374 (19%)	4.6	130	140	No	No
15	416 (16%)	5.4	140	163	Genital herpes	Yes
43	453 (16%)	4.6	150	163	Oral herpes	No
58	660 (29%)	2.6	260	257	No	No
16	686 (40%)	4.5	223	200	No	No
18	718 (23%)	5.4	237	230	Genital herpes	Yes
12	843 (26%)	4.5	243	253	Genital herpes	No
Mean of group	538 (26%)	4.6	184 ± 56	189 ± 49	6/9	3/9

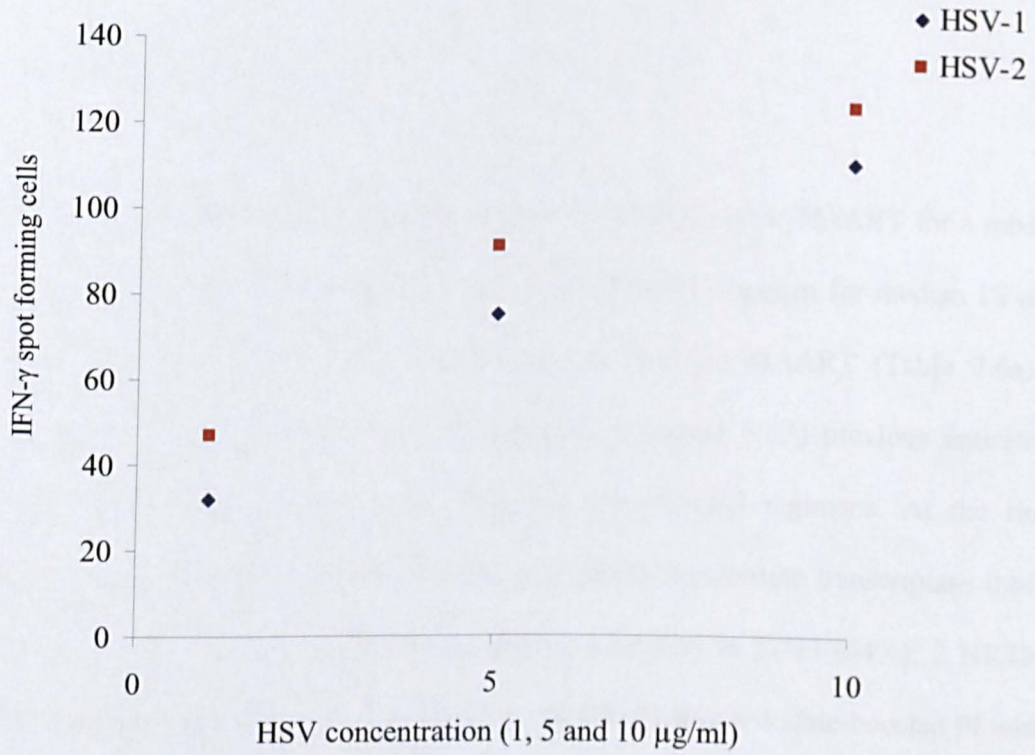


Figure 7.4. IFN-γ responses in a newly diagnosed HAART naïve patient

The plot illustrates IFN-γ responses to three different concentrations of HSV-1 and HSV-2 antigens. This patient had a CD4 count of 369 cells/mm^3 at the time of HIV-1 diagnosis. The patient had diagnosis of genital herpes and was receiving acyclovir at the time of sampling.

7.3.4.3 Patients on HAART

The study included 33 HIV-infected patients who had received HAART for a median of 30 months (range 1-109), including the current HAART regimen for median 12 months (range 1-60). Of these, 13/33 (39%) were on first-line HAART (Table 7.6a). The remaining 20 patients had experienced median 5 (range 3-13) previous antiretroviral drugs and median 3 (range 1-13) previous antiretroviral regimens. At the time of sampling, the HAART regimen included ≥2 NRTIs (nucleoside transcriptase inhibitor) and one NNRTI (non-nucleoside transcriptase inhibitor) in 21/33 (64%); 2 NRTIs and one ritonavir-boosted PI (protease inhibitor) in 8/33 (24%); a double-boosted PI with one NRTI or NNRTI in 3/33 (9%) and a triple NRTI in 1/33 (3%). Of the 33 patients, 28 (82%) patients had a HIV-1 plasma viral load <50 copies/ml and 30/33 (91%) ≤ 400 copies/ml.

Overall, 5/33 (15%) individuals reported a history of clinical HSV disease, including 3/5 cases of orolabial herpes and 2/5 cases of genital herpes and one patients was on acyclovir at the time of sampling. As shown in Tables 7.6a and 7.6b, overall, HSV-specific responses increased with CD4 counts. Patients 40 and 26 with the highest CD4 counts (1040 and 920 cells/mm^3 respectively) showed the strongest IFN-γ response (Table 7.6). Responses were not dissimilar in persons with or without clinical HSV disease (184 ± 62 versus 167 ± 59 SFC/10^6 PBMC for HSV-1 and 184 ±59 versus 157 ± 57 SFC/10^6 PBMC for HSV-2). Only one person was on acyclovir therapy.

Table 7.6a. IFN-γ responses to HSV-1 and HSV-2 in patients on first-line therapy

Patient	CD4 counts (cells/mm^3)	HIV plasma viral load (log_{10} copies/ml)	HAART regimen at time of sampling	Duration of HAART (months)	Mean IFN-γ SFC number		History of HSV disease†	On anti-HSV therapy at time of sampling
					HSV-1	HSV-2		
28	90 (7%)	4.8	3TC/DDI//EFV	2	73	63	Oral herpes	No
24	170 (10%)	<1.7	FTC/DDI/EFV	14	140	113	No	No
48	210 (8%)	<1.7	3TC/TDF/TPV/r	12	130	153	No	No
38	240 (15%)	<1.7	FTC/DDI/EFV	1	153	163	No	No
32	310 (14%)	<1.7	3TC/DDI//EFV	13	123	150	No	No
46	320 (21%)	<1.7	3TC/DDI//EFV	18	163	177	No	No
39	360 (30%)	<1.7	3TC/LPV/r/FPV	24	180	190	Oral herpes	No
51	420 (35%)	<1.7	FTC/DDI/NVP	15	190	190	No	No
19	470 (21%)	<1.7	FTC/TDF/EFV	7	230	183	Genital herpes	No
52	480 (18%)	<1.7	3TC/DDI//EFV	12	197	183	No	No
50	520 (19%)	<1.7	3TC/DDI//EFV	18	223	237	No	No
54	670 (32%)	1.8	3TC/DDI//EFV	13	257	250	No	No
26	920 (40%)	<1.7	3TC/DDI/NVP	28	290	303	No	No
Mean of group	398 (21)	11/13 <50 copies/ml		14	181 ± 59	181 ± 60	3/13	0/13

Table 7.6b. IFN-γ responses to HSV-1 and HSV-2 in drug-experienced patients on HAART

Patient	CD4 counts (cells/mm^3)	HIV plasma viral load (log_{10} copies/ml)	HAART regimen at time of sampling	Duration of current HAART (months)	Total months of therapy	Mean IFN-γ SFC number		History of HSV disease[†]	On anti-HSV therapy at time of sampling
						HSV-1	HSV-2		
45	90 (10%)	5.0	3TC/TDF/LPV/r	17	71	70	100	No	No
49	170 (10%)	<1.7	3TC/TDF/EFV	26	30	110	133	No	No
42	200 (23%)	2.0	TDF/DDI/ATV/r	5	23	150	147	No	No
30	200 (19%)	<1.7	3TC/DDI/EFV	7	17	123	110	No	No
56	210 (9%)	<1.7	TDF/ABC/ATV/r	8	23	90	83	No	No
53	220 (12%)	<1.7	3TC/DDI//EFV	3	57	137	120	No	No
25	240 (17%)	4.0	TDF/LPV/r/SQV	10	90	220	223	No	No
55	270 (14%)	<1.7	FTC/TDF/LPV/r	5	41	107	130	No	No
2	282 (19%)	<1.7	NVP, LPV/r/SQV	12	69	157	170	Genital herpes	No
31	350 (27%)	<1.7	3TC/TDF/EFV	14	56	173	167	No	No
23	370 (15%)	<1.7	TDF/d4T/ATV/r	7	70	197	180	Oral herpes	Yes
36	420 (20%)	<1.7	3TC/DDI//EFV	21	45	200	193	No	No
57	430 (20%)	<1.7	3TC/ABC/AZT/NVP	60	74	173	197	No	No
6	470 (23%)	<1.7	TDF, AZT, ABC	33	45	223	237	No	No
34	530 (22%)	<1.7	3TC/TDF/NVP	2	34	217	203	No	No
60	590 (23%)	<1.7	TDF/ABC/ATV/r	8	67	203	223	No	No
59	610 (25%)	<1.7	3TC/ABC/EFV	11	44	250	230	No	No
20	780 (43%)	<1.7	TDF/3TC/EFV	16	58	250	237	No	No
22	970 (29%)	<1.7	3TC/TDF/NVP	24	84	277	257	No	No
40	1040 (30%)	<1.7	3TC/TDF/ATV/r	6	109	303	297	No	No
Mean of group	422 (21%)	17/20 (50 copies/ml		15	55	182 ± 64	180 ± 58	2/20	1/20

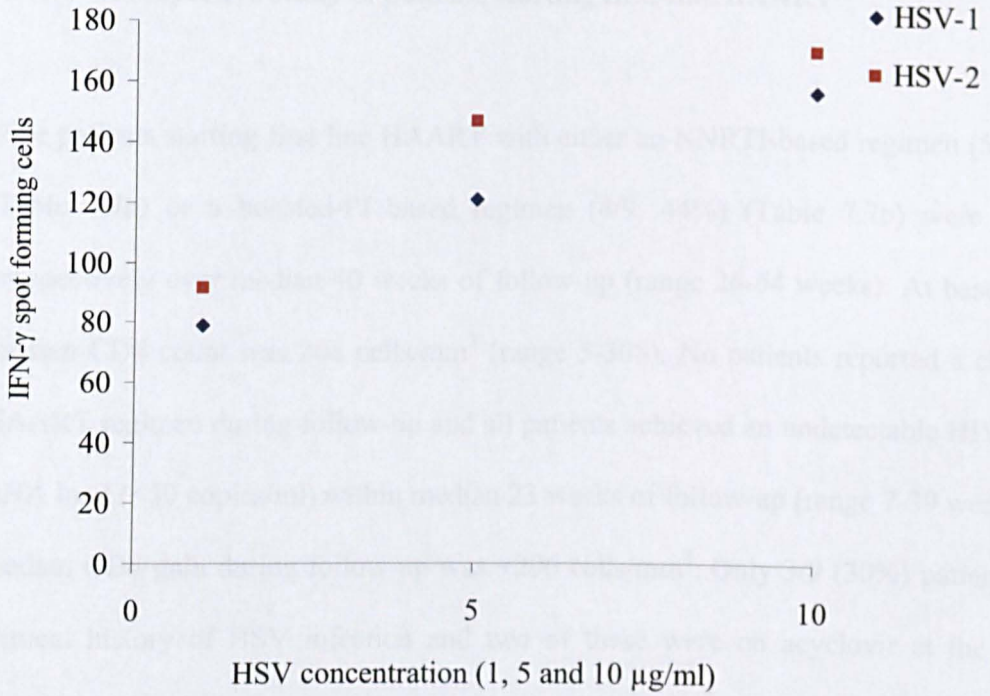


Figure 7.5. IFN-γ responses to HSV-1 and HSV-2 in a HAART treated patient

The plot illustrates IFN-γ responses to three different concentrations of HSV-1 and HSV-2 antigens. The patient was receiving therapy with NVP, SQV, LPV/r and showed a CD4 count of 282 cells/mm^3. He had a history of genital herpes but was not on acyclovir at the time of sampling.

7.3.4.4 Prospective study of patients starting first-line HAART

Nine patients starting first line HAART with either an NNRTI-based regimen (5/9, 56%) (Table 7.7a) or a boosted-PI based regimen (4/9, 44%) (Table 7.7b) were sampled prospectively over median 40 weeks of follow-up (range 26-64 weeks). At baseline, the median CD4 count was 208 cells/mm^3 (range 5-308). No patients reported a change of HAART regimen during follow-up and all patients achieved an undetectable HIV plasma RNA load (<50 copies/ml) within median 23 weeks of follow-up (range 7-39 weeks). The median CD4 gain during follow-up was +296 cells/mm^3. Only 3/9 (30%) patients had a clinical history of HSV infection and two of these were on acyclovir at the time of sampling.

In five patients (no 5, 7, 27, 3 and 13) with low or absent HSV responses at baseline, HAART-induced increases in CD4 cells were accompanied by parallel increases in the mean number of SFCs. In this group the median baseline CD4 count was 189 cell/mm^3 (range 5-236) and increased by +202 cells/mm^3 (range 26-559) during median 48 weeks (39-60) of follow-up. The median SFC increase was +72/10^6 PBMC (range 23-187). The IFN-γ response to HSV-1 and HSV-2 before and after the initiation of HAART are plotted in Figures 7.6, 7.7a-7.7j. Responses remained stable in 2 other patients (9 and 37). One of the two showed a substantial increase in CD4 counts (+357 cells/mm^3) over 38 weeks of follow-up. However in the 2 individuals (9 and 37), a reduction in HIV viral load (50 copies/ml) was not achieved until week 38. In the other patient, the CD4 count did not improve during 26 weeks of follow-up, despite the patient demonstrating

undetectable viral load by week 15. Finally, two patients (17 and 37) with strong HSV responses at baseline showed a modest decline in SFC number over time.

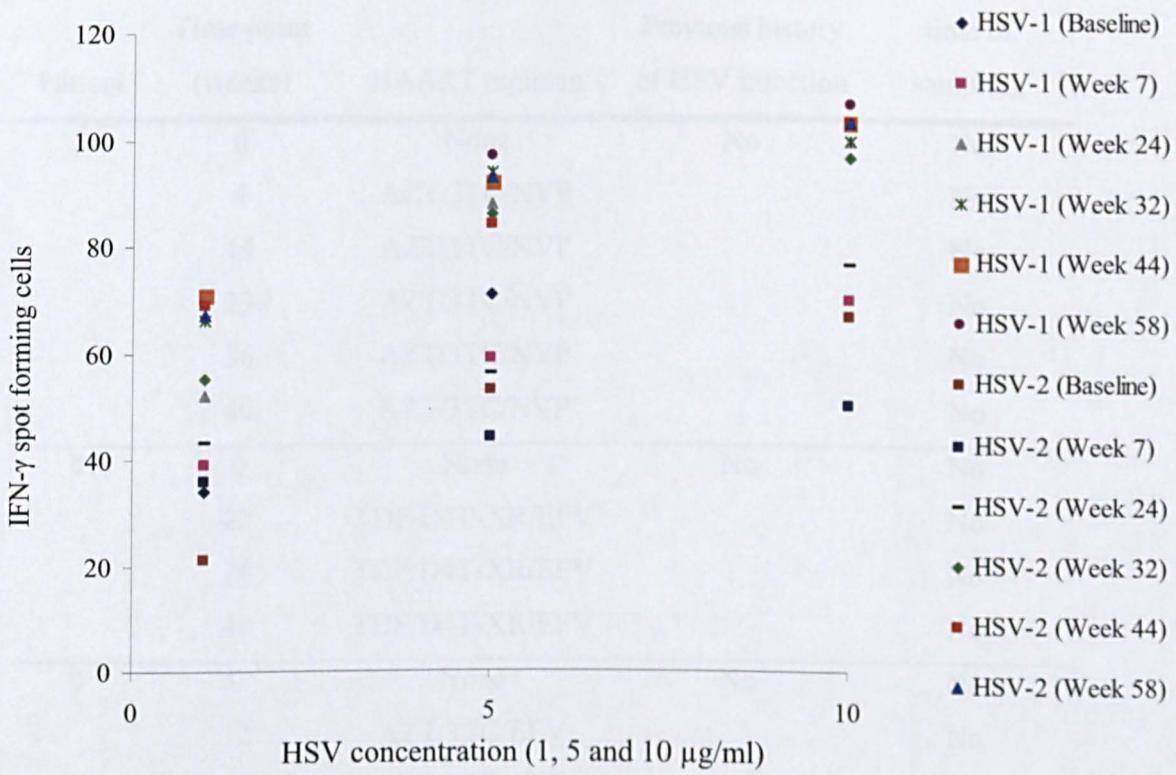


Figure 7.6. IFN-γ responses to HSV-1 and HSV-2 in a prospective patient before and after the initiation of HAART

The plot illustrates IFN-γ responses to three different concentrations of HSV-1 and HSV-2 antigens. This patient exhibited a CD4 count of 107 cells/mm^3 at baseline which increased to 169 cells/mm^3 at week 58. The patient also had a clinical history of genital herpes and was on acyclovir therapy.

Table 7.7a. Prospective IFN-γ responses to HSV-1 and HSV-2 in patients both before and after the initiation of NNRTI-based HAART

Patient	Time point (weeks)	HAART regimen	Previous history of HSV infection	On anti-HSV therapy at time of sampling
5	0	None	No	No
	4	AZT/3TC/NVP		No
	14	AZT/3TC/NVP		No
	23	AZT/3TC/NVP		No
	36	AZT/3TC/NVP		No
	40	AZT/3TC/NVP		No
7	0	None	No	No
	22	TDF/D4T-XR/EFV		No
	26	TDF/D4T-XR/EFV		No
	48	TDF/D4T-XR/EFV		No
9	0	None	No	No
	12	AZT/3TC/EFV		No
	31	AZT/3TC, EFV		No
	38	AZT, 3TC, EFV		No
13	0	None	Genital herpes	Yes
	7	TDF/D4T-XR/EFV		No
	24	TDF/D4T-XR/EFV		No
	32	TDF/D4T-XR/EFV		No
	44	TDF/D4T-XR/EFV		No
	58	TDF/D4T-XR/EFV		No
37	0	None	No	No
	15	AZT/3TC/NVP		No
	26	AZT/3TC/NVP		No

Table 7.7b. Prospective IFN-γ responses to HSV-1 and HSV-2 in patients both before and after the initiation of PI-based HAART

Patient	Time point (weeks)	HAART regimen	Previous history of HSV infection	On anti-HSV therapy at time of sampling
27	0	None	Oral herpes	Yes
	4	TDF/3TC/LPV/r		No
	11	TDF/3TC/LPV/r		No
	30	TDF/3TC/LPV/r		No
	39	TDF/3TC/LPV/r		No
8	0	None	No	No
	18	AZT/3TC/LPV/r		No
	36	AZT/3TC/LPV/r		No
	53	AZT/3TC/LPV/r		No
	64	AZT/3TC/LPV/r		No
3	0	ABC/3TC,/ATV/r	No	No
	23	ABC/3TC,/ATV/r		No
	35	ABC/3TC,/ATV/r		No
	48	ABC/3TC,/ATV/r		No
	60	ABC/3TC,/ATV/r		No
17	0	None	Genital herpes	No
	16	TDF/3TC/LPV/r		No
	25	TDF/3TC/LPV/r		No
	32	TDF/3TC/LPV/r		No

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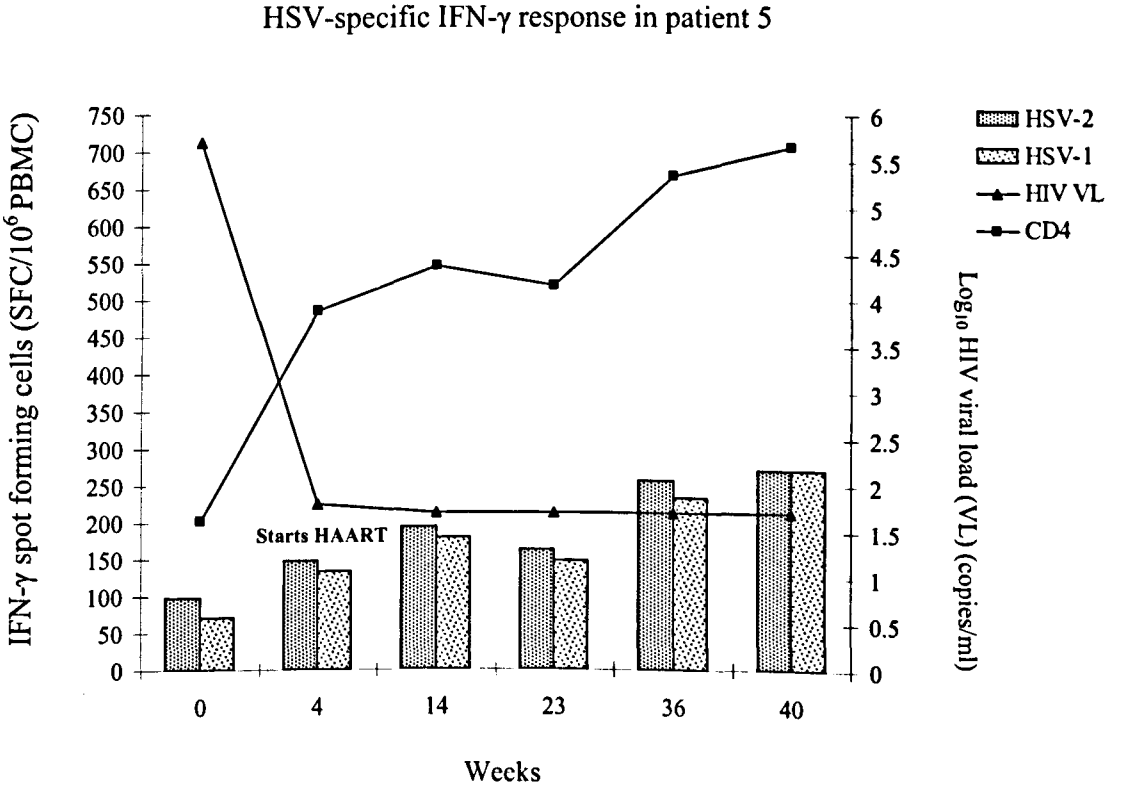
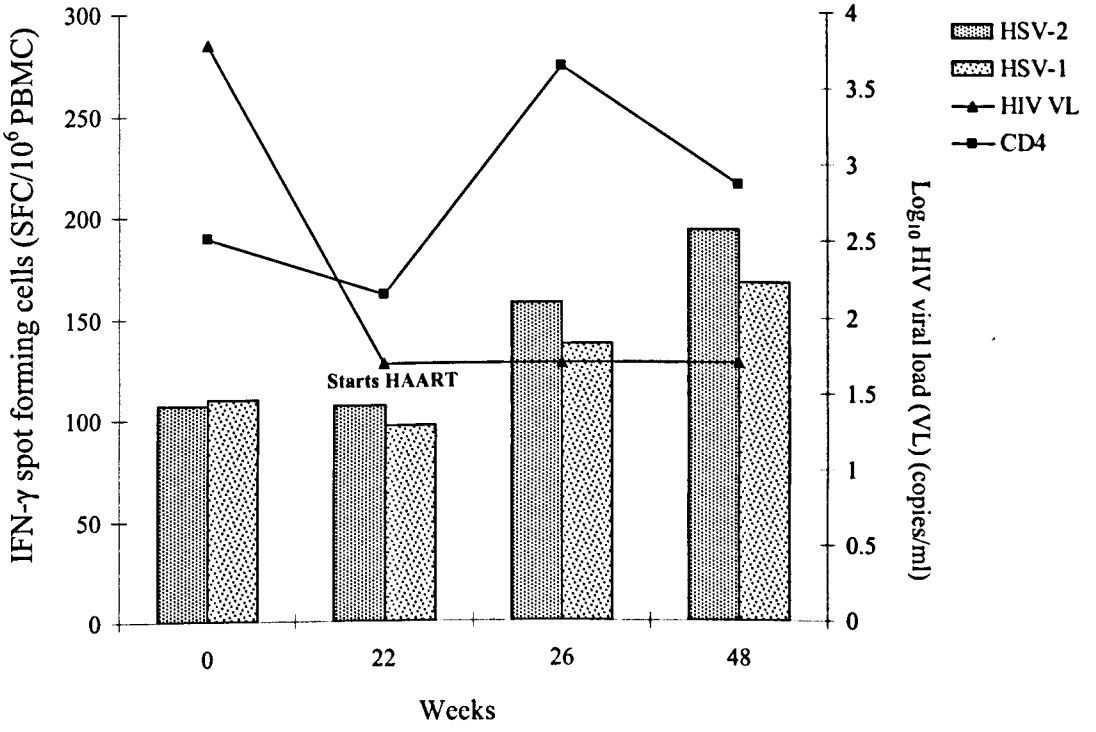
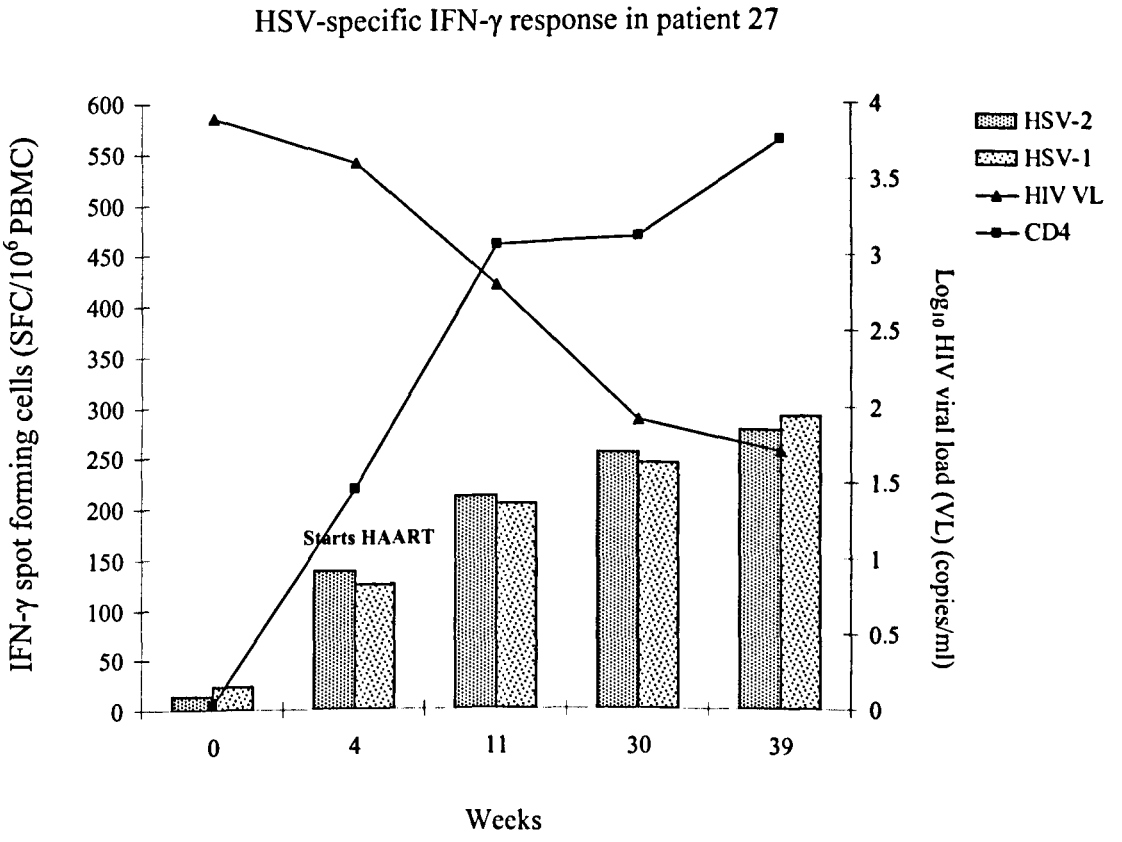

HSV-specific IFN-γ response in patient 5
IFN-γ spot forming cells (SFC/10^6 PBMC)
Log10 HIV viral load (VL) (copies/ml)
HSV-2
HSV-1
HIV VL
CD4
Starts HAART
Weeks
0
4
14
23
36
40

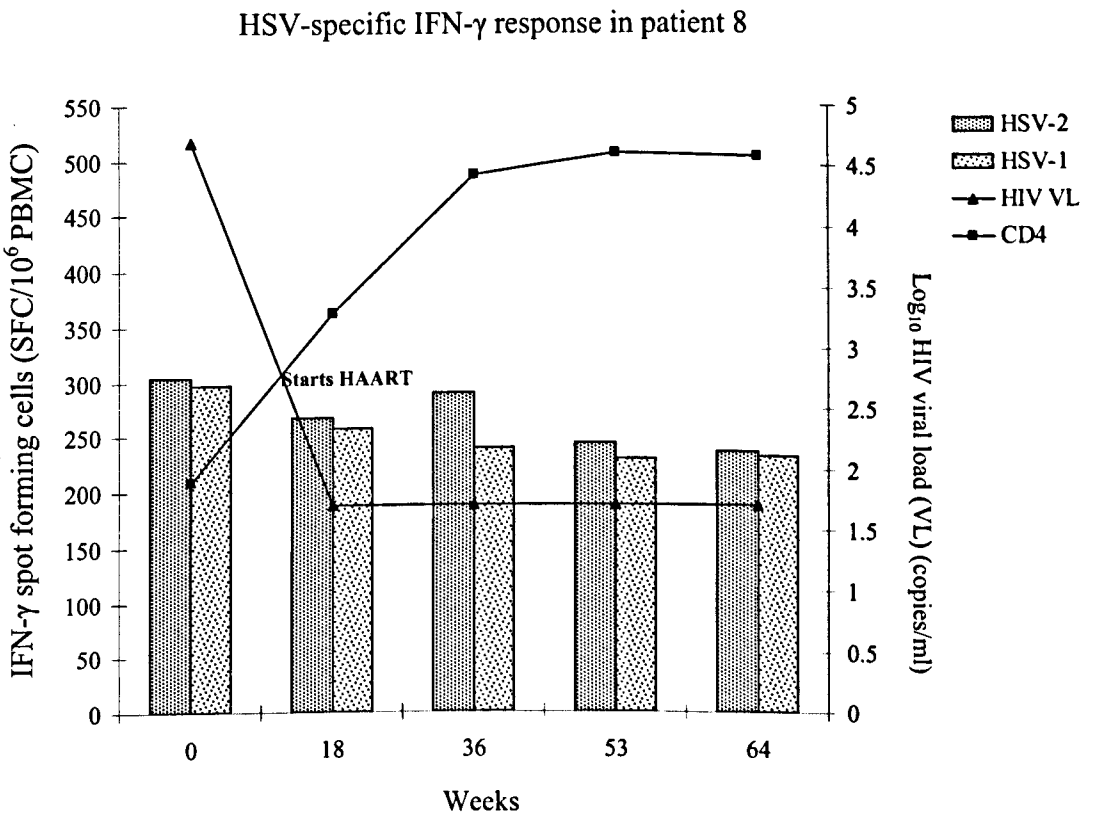
b


HSV-specific IFN-γ response in patient 7
IFN-γ spot forming cells (SFC/10^6 PBMC)
300
250
200
150
100
50
0
Starts HAART
0
22
26
48
Weeks
4
3.5
3
2.5
2
1.5
1
0.5
0
Log10 HIV viral load (VL) (copies/ml)
HSV-2
HSV-1
HIV VL
CD4

c


HSV-specific IFN-γ response in patient 27
IFN-γ spot forming cells (SFC/10^6 PBMC)
Log10 HIV viral load (VL) (copies/ml)
HSV-2
HSV-1
HIV VL
CD4
Starts HAART
600
550
500
450
400
350
300
250
200
150
100
50
0
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
11
30
39
Weeks

d


HSV-specific IFN-γ response in patient 8
IFN-γ spot forming cells (SFC/10^6 PBMC)
Log10 HIV viral load (VL) (copies/ml)
Starts HAART
HSV-2
HSV-1
HIV VL
CD4
Weeks
0
18
36
53
64
550
500
450
400
350
300
250
200
150
100
50
0
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0

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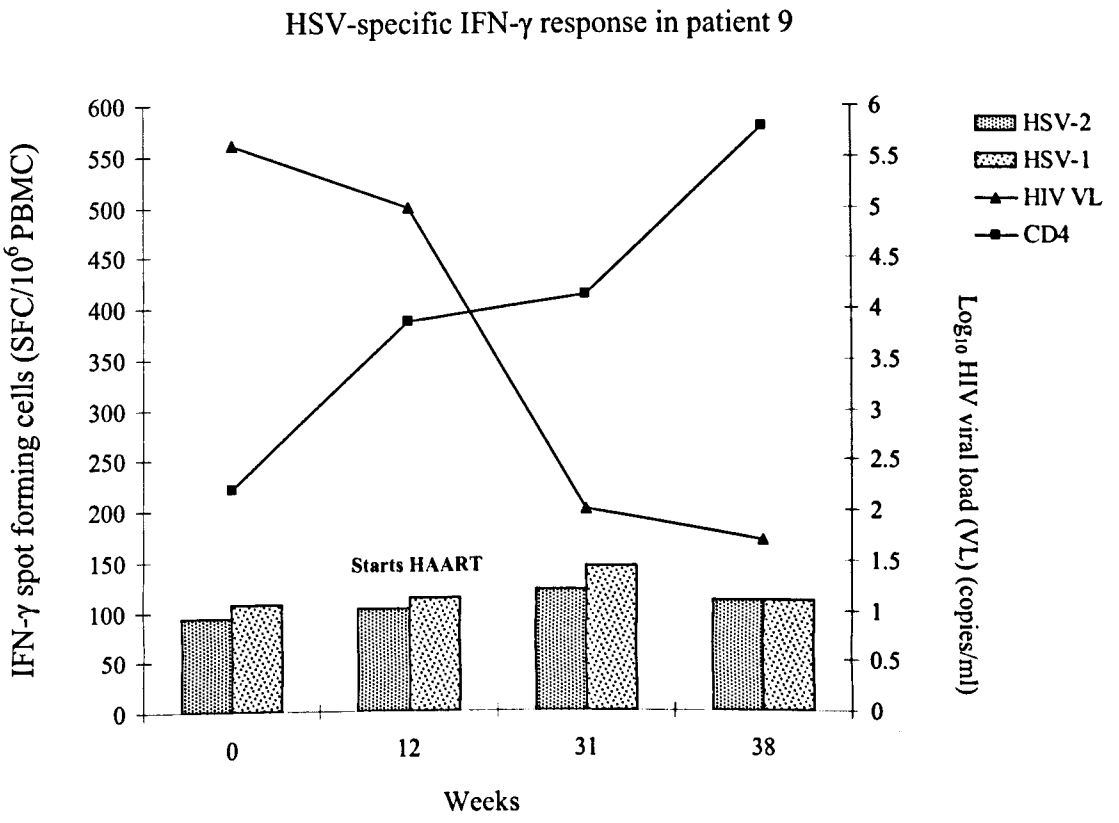
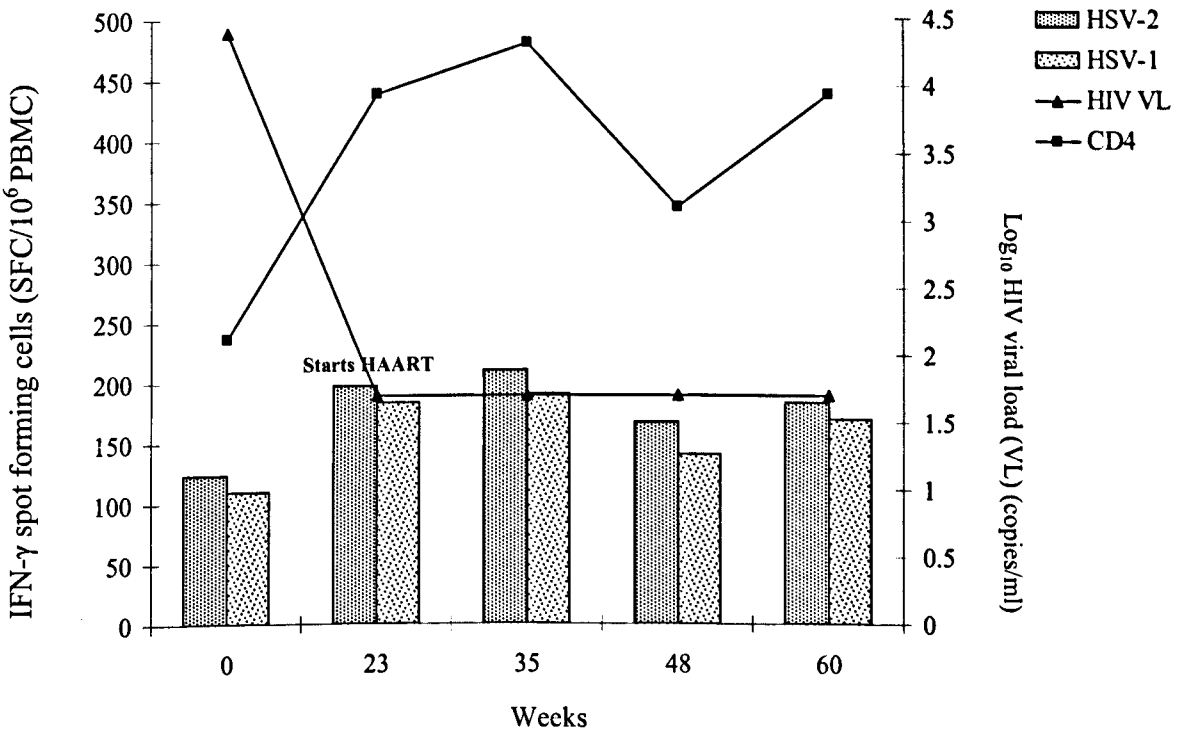
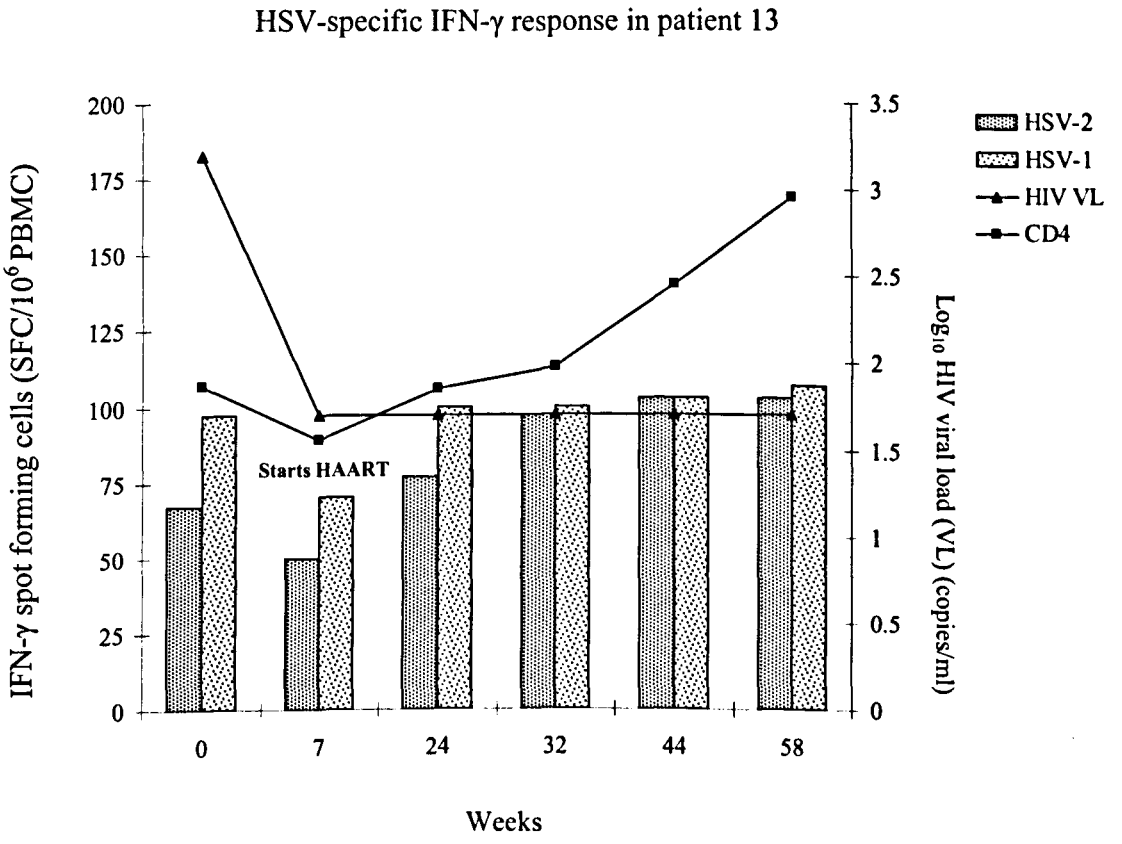

HSV-specific IFN-γ response in patient 9
IFN-γ spot forming cells (SFC/10^6 PBMC)
600
550
500
450
400
350
300
250
200
150
100
50
0
Starts HAART
0
12
31
38
Weeks
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Log10 HIV viral load (VL) (copies/ml)
HSV-2
HSV-1
HIV VL
CD4

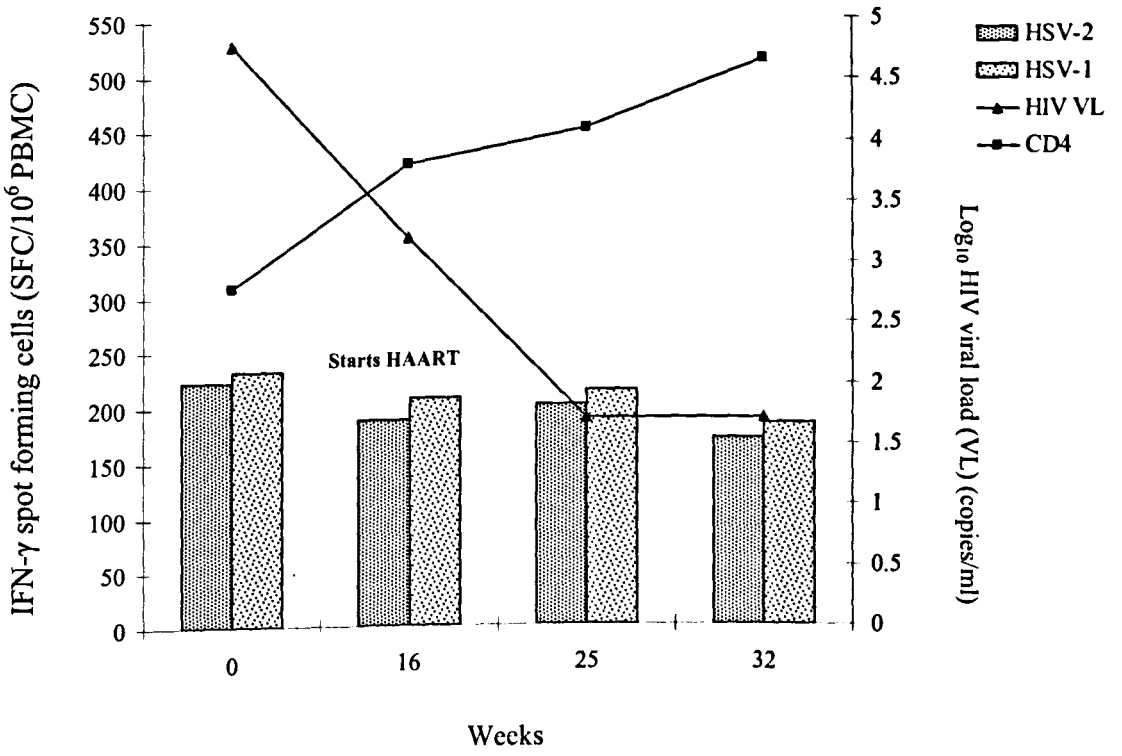
f


HSV-specific IFN-γ response in patient 3
IFN-γ spot forming cells (SFC/10^6 PBMC)
Log10 HIV viral load (VL) (copies/ml)
Starts HAART
Weeks
0
23
35
48
60
HSV-2
HSV-1
HIV VL
CD4

g


HSV-specific IFN-γ response in patient 13
IFN-γ spot forming cells (SFC/10^6 PBMC)
200
175
150
125
100
75
50
25
0
3.5
3
2.5
2
1.5
1
0.5
0
Log10 HIV viral load (VL) (copies/ml)
HSV-2
HSV-1
HIV VL
CD4
Starts HAART
0
7
24
32
44
58
Weeks

h


HSV-specific IFN-γ response in patient 17
IFN-γ spot forming cells (SFC/10^6 PBMC)
550
500
450
400
350
300
250
200
150
100
50
0
Starts HAART
0
16
25
32
Weeks
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Log10 HIV viral load (VL) (copies/ml)
HSV-2
HSV-1
HIV VL
CD4

i

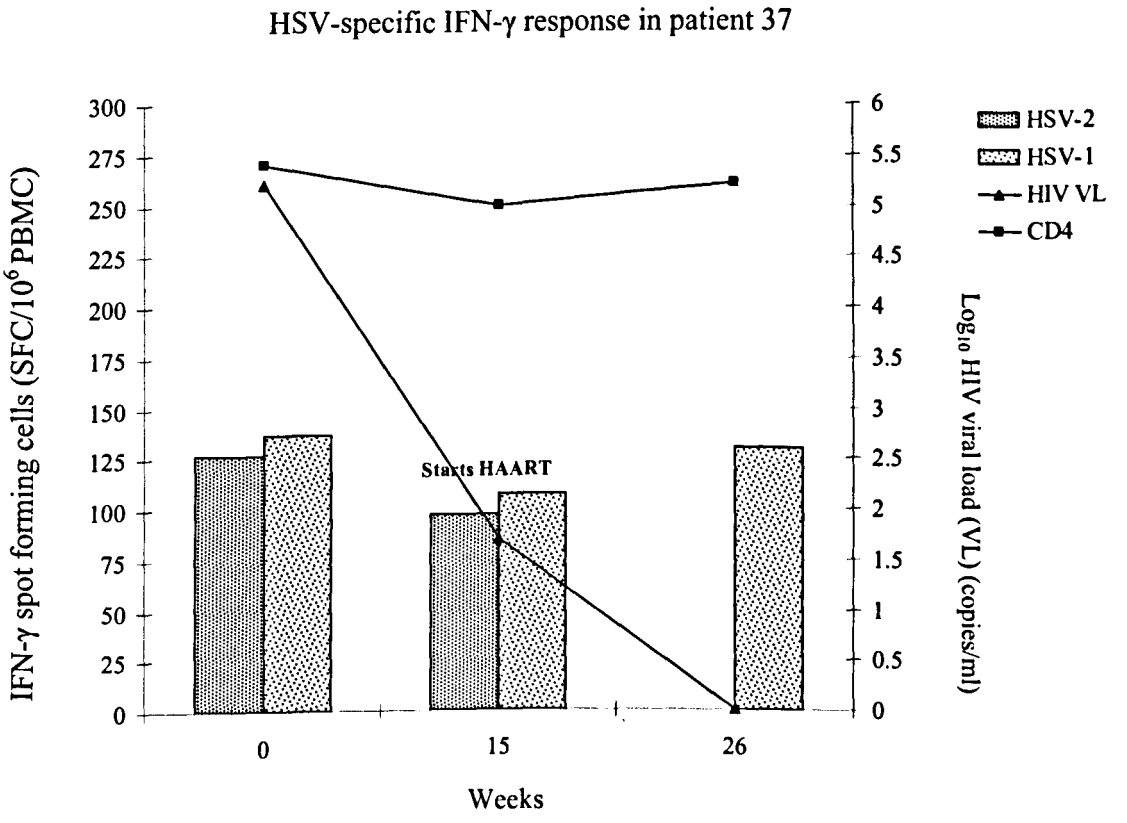


Figure 7.7a-7.7i. HSV specific IFN-γ responses among patients before and after the initiation of HAART. IFN-γ responses to HSV-1 and HSV-2 lysates are plotted for each of the 9 patients before and after commencing HAART. The initial sample at baseline is referred as week 0, and sequential samples thereafter post HAART.

7.3.5 Correlation between HSV responses and CD4 counts in HSV IgG seropositive HIV-1 infected patients

Mean IFN-γ responses for HSV-1 and HSV-2 were similar among the 54 HIV-1 infected individuals (189 versus 187 SFC/10^6 PBMC) (Figure 7.9). The coefficient of correlation was 0.75.

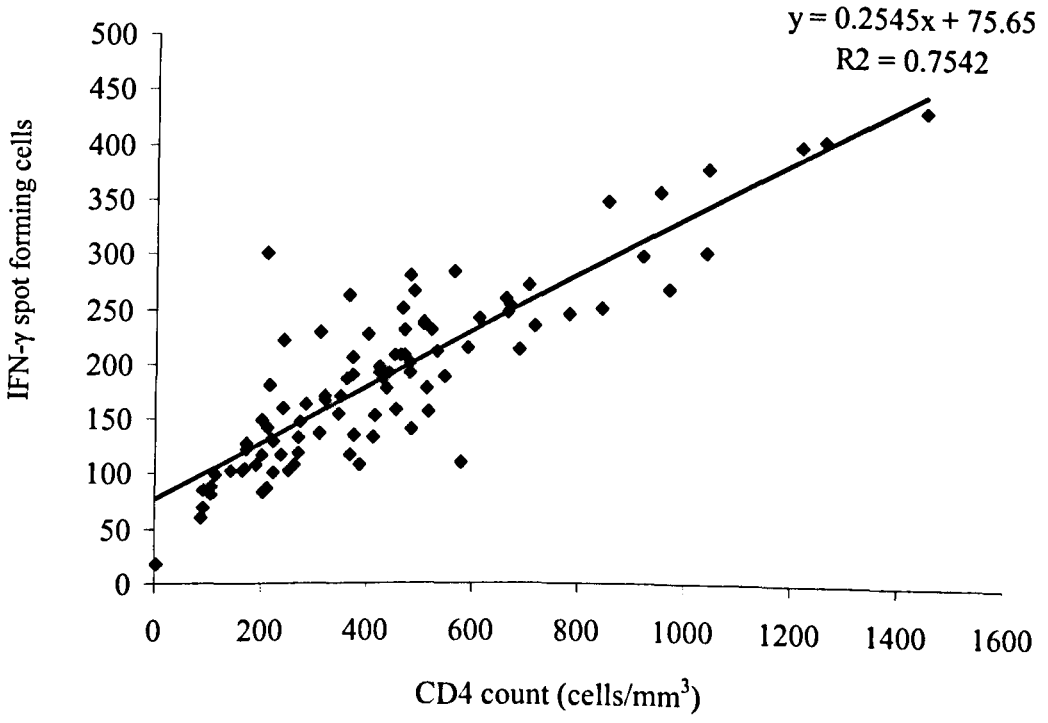


Figure 7.8. Correlation between CD4 count and HSV-specific IFN-γ responses among HIV-1 infected HSV IgG seropositive patients. IFN-γ responses to HSV-1 and HSV-2 lysates (averaged from HSV-1 and HSV-2 data) are plotted for the 54 patients.

7.4 DISCUSSION

Highly active anti-retroviral therapy has shown to restore $CD4^+$ T-cell counts in HIV-1 infected individuals (Jevtovic *et al.,* 2005). However, there are limited data on HSV specific CD4 T-cell responses among HIV-1 infected subjects at different stages of HIV-1 disease and on the effects of HAART on immune reconstitution of HSV-specific T-cell responses. In the present study, we examined HSV-specific T cell responses among HIV-1 infected patients, using IFN-γ as an immunological marker following HSV-1 and HSV-2 antigenic stimulation of PBMC *in vitro*. We present data within 4 distinct groups of the HIV-1 infected population, each group representing individuals at different clinical stages of HIV-1 infection: prospective patients starting HAART; long-term slow and non progressors; newly diagnosed HIV-1 infected patients not receiving HAART; and patients on stable HAART. Results demonstrated that HSV-specific T-cell mediated production of IFN-γ correlated with CD4 cell counts. Responses were highest in patients who had elevated CD4 cell counts associated with LTNP/LTSP status, early HIV infection or HAART-induced CD4 restoration. Taking the mean HSV-specific response of healthy HSV IgG seropositive donors as a reference (318 ± 90 SFC/10^6 PBMC), a CD4 count >500 cells/mm^3 was generally required to achieve comparable responses in HIV-infected patients.

Patients with long-standing infection in the absence of clinical progression showed HSV responses comparable to healthy HIV-1 uninfected controls, consistent with the preserved immune function that characterises these patients. Previous observations have indicated

that LTNP control viremia with no diminution of CD4 cells (Sankaran *et al.,* 2005; Valdez *et al.,* 2002; Paroli *et al.,* 2001). In addition, LTNP may remain clinically healthy for >7 years in the absence of HAART (Sankaran *et al.,* 2005). Evidence suggests that these patients have vigorous HIV-1 specific CD4 T cell responses with high levels of IFN-γ production (Paroli *et al.,* 2001). However, after several years of HIV-1 infection, evidence of disease progression may start to appear reflected by a decline in CD4 cell counts, so that at least some of these patients are better defined as Long-term slow progressors (LTSP). These observations are consistent with findings from patient 21, who after 19 years since the diagnosis had a CD4 count of 480 cells/mm^3 but lost 110 cells during 30 weeks of follow up.

Data (Lundgren *et al.,* 2002; Ghani *et al.,* 2001; Lewden *et al.,* 2002) has indicated that the CD4 count acts as a strong predictor of clinical outcome in HIV infected patients. HAART has been previously shown to effectively restore CD4 counts and reconstitute immune functions in HIV-1 infected individuals (RD *et al.,* 2005; Anastos *et al.,* 2004; Servais *et al.,* 2000). In a study of 377 HIV-1 infected women in resource limited settings who initiated HAART with pre-HAART CD4 counts <200 cells/mm^3, 43% exhibited CD4 counts >200 and 11% >350 cells/mm^3 within 1 year of starting HAART (Anastos *et al.,* 2004). The authors also reported reduction of AIDS-defining illness among women to be associated with increased CD4 counts >350 cells/ mm^3. However, data on delayed type hypersensitivity (DTH) responses from this study was limited since factors other than HIV-1 infection could impair DTH responses and that variability in DTH itself can exist over short time periods (1 year).

A recent study of 153 HAART naïve patients demonstrated mean CD4 count increases of 149 and 204 cells/mm^3 at 24 weeks and 48 weeks respectively after the initiation of HAART, paralleled with a reduction in the patient's HIV-1 viral load (Wester *et al.*, 2005). Previous studies have reported CD4 counts to increase at least 50 cells/mm^3 after 4-8 weeks of initiating HAART, followed by an increase of 50-100 cells/mm^3 per year thereafter (Yamashita *et al.*, 2001; Le Moing *et al.*, 2002).

These observations are in agreement with our findings. Among the 9 individuals who started first line HAART with either NNRTI or boosted PI-based-regimens, HIV-1 RNA load was reduced to undetectable levels (<50 copies/ml) within median 23 weeks. The overall CD4 increase in the cohort was median +296 cells/mm^3 over median 40 weeks of follow-up. One patient (n=27) demonstrated a dramatic increase of +213 cells/mm^3 after just 4 weeks of HAART and of +455cells/mm^3 after 11 weeks followed by a slower subsequent increase. Initial rises in CD4 count after 1-2 months of therapy have been shown to occur as a result of redistribution of cells from the bone marrow (Bucy *et al.*, 1999; Michael *et al.*, 2002), but this data must be interpreted with caution since only 7 HIV-1 infected patients were followed up (Bucy *et al.*, 1999). This may be followed by a decline in CD4 cell numbers followed by a second, slower rise with continued suppression of viral replication, which may continue for >12 months (Wu *et al.*, 2001; Kaufmann *et al.*, 1998).

The poor HSV-specific response observed in the newly diagnosed HAART naïve subjects, suggests a relationship between the inability to produce IFN-γ following HSV stimulation and dysfunctionality of CD4+ T cells (Hardy *et al.*, 2003). Conversely, the recovery of IFN-γ responses after commencing HAART in patients with low or absent responses at baseline is in agreement with observations from other studies that have demonstrated increased proliferative responses to HSV antigenic stimulus following the initiation of HAART (Hardy *et al.*, 2003).

Clinical manifestations of HSV infection were overall uncommon in the HIV-infected population, with 14/54 (26%) patients having a recognised clinical history of orolabial or genital HSV disease. These findings are in contrast with the recognised association between HIV infection and HSV disease burden, and are likely to reflect the beneficial effects of HAART in restoring HSV specific T-cell responses. In a study of 28 HAART treated and 49 HAART naive persons co-infected with HIV-1 and HSV-2, HAART treated individuals reportedly had HSV lesions on fewer days with lower HIV viral load compared to drug naïve individuals (2.8% versus 11.3% of days respectively; 170 copies/ml versus 18793 copies/ml respectively) (Posavad *et al.*, 2004). This is in agreement with the findings reported in chapter 6, which showed that HIV-1 infected patients diagnosed with genital herpes after 1997 were four times less likely to receive a clinical diagnosis of genital herpes than patients diagnosed in the pre-HAART era (adjusted odds ratio 5.11; 95% CI: 3.28-7.98; P=0.0001) (Ramaswamy *et al.*, 2006).

Findings from this study have shown that HAART improves the clinical course of HSV infection. Conversely, other studies have also shown that immune reconstitution following HAART may aggravate the immunopathology of HSV and other opportunistic infections (Fox *et al.,* 1999; Leidner & Aboulafia, 2005; Goebel, 2005; Shelburne *et al.,* 2005; Buckingham *et al.,* 2004). To date, there is only one study which has documented the adverse effect of immune reconstitution by HAART resulting in chronic erosive HSV infection of the penis in a male patient within 4-6 weeks after commencing HAART (Fox *et al.,* 1999). It has been postulated that HSV was present prior to the initiation of HAART and that the recovery of the immune system following HAART, resulted in a hypersensitivity response leading to immune restoration disease (IRD). No HSV-related IRD was observed in this study.

The results support the consideration that HAART enables immunological reconstitution of CD4 cells and HSV specific responses. This can be further demonstrated by improved control of HSV clinical disease as observed in our cohort of HAART-treated patients. In addition, HSV-specific immune responses comparable to those of healthy donors were only evident at CD4 cell counts above 500 cells/mm^3. This provides a threshold for HSV-immune reconstitution that may assist with clinical management.

8. Chapter 8. General Discussion

Despite the availability of effective antiviral therapy to control and possibly prevent infection, genital herpes is increasingly common in both developed and developing countries. The work described in this thesis addressed the following topics: 1) the optimal diagnostic strategies for genital herpes; 2) the epidemiology of genital herpes in an ethnically diverse population of GUM attendees including an analysis of virus genetic variability 3) the epidemiology and natural history of HSV-2 infections in a cohort of HIV-1 infected individuals, and 4) the impact of HAART on HSV-specific T-cell mediated immune responses.

The optimal diagnostic strategies for genital herpes

The feasibility of using real-time PCR as a diagnostic tool for detecting genital herpes in genital swabs was investigated among GUM clinic attendees with suspected genital herpes (Chapter 3a). Results demonstrated real-time PCR to have a significantly higher diagnostic yield compared to virus culture in patients who presented early after the onset of symptoms, and had visible genital ulceration. These findings were consistent with those from other studies and supported the use of PCR as the preferred diagnostic tool (Sharon *et al.*, 1997; Cone *et al.*, 1991; Rand *et al.*, 2005; Scoular *et al.*, 2002; Schmutzhard *et al.*, 2004).

One of the potential limitations of PCR is the risk of false negative and false positive results. False negative results may be the consequence of inhibitors present within the sample (Bezold *et al.*, 2000). We sought to detect inhibitory substances when developing our LightCycler PCR assay by spiking HSV negative samples with HSV-1 and HSV-2 DNA, however no significant inhibition was found. In addition no sample that tested positive by virus culture failed to be detected by PCR. We also compared the three specimen preparation methods for PCR to determine the one that allowed optimal virus detection. The three methods showed a high level of concordance, although there were a few specimens positive by PEG/NaCl precipitation and negative by manual and automated DNA extraction. By adapting the PEG virus precipitation method for HSV detection, we enhanced the diagnostic feasibility of real-time PCR for HSV detection by decreasing costs and time required for specimen preparation. Further studies are ongoing to ascertain whether it may be possible to retain high diagnostic sensitivity while avoiding specimens preparation altogether.

Additional information for patient management and counselling can be provided by HSV typing. The HSV LightCycler PCR protocol allowed HSV typing by melting curve analysis. The level of sequence conservation for the hybridisation probe targets is critical for the success of melting curve analysis (Whiley *et al.*, 2004). It was interesting to note that although the PCR assay was specific in HSV type discrimination, atypical melting curves were apparent in 14 (18%) HSV-2 positive specimens. Consistent with earlier observations (Anderson *et al.*, 2003; Issa *et al.*,

2005), nucleotide differences in the probe binding region of the DNA polymerase gene was observed in 4/14 (29%) specimens. One novel observation was that melting curves were influenced by specimen preparation methods, in the presence of conserved gene sequences.

The application of HSV type specific serology was investigated to determine its potential contribution to the diagnosis of genital herpes. Commercially available tests accurately discriminate between HSV-1 and HSV-2 antibodies and are based on HSV type-specific sequences within the viral glycoprotein G. We optimised and evaluated a commercially available HSV type-specific EIA to accurately identify HSV-1 and HSV-2 antibodies in several defined populations. Our evaluation studies indicated that increasing the manufacturer's recommended cut-off from 1.1 to 3.1 improved specificity without compromising sensitivity. This was particularly evident in patient sera from sub- Uganda and Kenya.

The observation that HSV EIA and Immunoblot can be successfully employed to determine newly acquired HSV-2 infection has been previously documented (Ashley-Morrow *et al.*, 2003; Howard *et al.*, 2003; Ashley, 2002). We demonstrated HSV-2 seroconversion in two clinical cases, including a case of HSV-2 encephalitis which showed HSV-2 seroconversion within 4 weeks of onset of symptoms. These findings supported previous observations, which suggested the median time of HSV-2 seroconversion was 21-23 days after the onset of symptoms.

Among patients who presented with genital herpes, HSV type-specific serology detected HSV-2 antibodies in all patients with recurrent genital herpes and with a HSV-2 positive genital swab. However, among patients with first-episode genital herpes and a HSV-2 positive, 38% were HSV-2 seropositive at time of presentation. This indicated the infection to be recurrent. Hence, the correct characterisation of patients presenting with genital herpes cannot rely on clinical grounds alone. We also observed 37% of patients clinically diagnosed with genital herpes with negative genital swab by PCR to be HSV-2 seropositive. This suggested that even when using PCR as a diagnostic tool, type-specific serology can assist with the diagnostic process in patients suspected of having genital herpes.

The epidemiology of genital herpes in an ethnically diverse population if GUM attendees

Genital ulceration is most commonly caused by Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (Mertz, 1990). The natural history of HSV-1 and HSV-2 infection differs particularly in the likelihood of recurrences. HSV-2 is five times more likely to cause recurrent disease than HSV-1 (Engelberg *et al.*, 2003). Traditionally HSV-2 has been associated with genital infection and HSV-1 with oral mucocutaneous disease. However, published studies have reported HSV-1 to account for over 50% of cases of genital herpes in the UK (Ross *et al.*, 1993; Scoular *et al.*, 2002; Slomka *et al.*, 1998; Lamey & Hyland, 1999). We used real-time PCR to explore the epidemiology of HSV genital infection in a cohort of GUM attendees

suspected of having genital herpes (Chapter 4). A clinical diagnosis of first-episode and recurrent genital herpes was made in 61% and 39% of individuals respectively.

HSV DNA was detected in 58% of genital swabs by real-time PCR. In contrast with other published studies in the UK (Coyle *et al.*, 2003; Ross *et al.*, 1993; Tayal *et al.*, 1994; Manavi *et al.*, 2004), we found that HSV-2 accounted for 93.5% of cases of HSV genital infection. However, results from these published studies must be interpreted with caution, due to the lack of demographic data. Interestingly in our study, HSV type associated with genital infection was influenced by ethnicity. Persons of white ethnicity were more likely to be infected with HSV-1 and persons of black ethnicity were more likely to be infected with HSV-2. Most studies to date have documented initial HSV-1 genital-tract infection to be prevalent in persons of white ethnicity than non-white ethnicities (Siegel *et al.*, 1992; Nageswaran *et al.*, 1996; Schillinger *et al.*, 2004). Possible explanations could be that oropharyngeal HSV-1 infection in childhood may play a role in determining the risk of genital infection with HSV-1 in adult life. In addition, it could be proposed that patients of black ethnicity were more reluctant than patients of white ethnicity to attend the GUM clinic if infected with HSV-1. The levels of oropharyngeal HSV-1 infection remain high in much of the developing world and are generally higher in poor social strata of the population. In contrast, the infection is becoming less common among affluent populations in developed countries which could be attributed to differences in sexual behaviour between different ethnicities (Lafferty, 2002).

A significant difference in the delay between the onset of symptoms and clinical presentation was also observed between men and women. Earlier presentation of HSV infection women compared to men may relate to biological factors such as the larger and more vulnerable mucosal surface of women causing intense pain (Carpenter *et al.*, 1999; Nicolosi *et al.*, 1994). Differences in awareness and reporting of symptoms or greater ease of accessing medical care may also be a contributing factor.

HIV testing was offered routinely to all patients presenting to the GUM clinic, but was declined by a large proportion of persons of black-Caribbean origin. This was worrying finding considering the high incidence of HIV in black-Caribbean populations (McBride *et al.*, 2005; Fenton *et al.*, 2005).

The variability of the HSV-2 UL 14 gene in patients clinically diagnosed with genital herpes and a positive genital swab was also studied using PCR (Chapter 4). Besides showing a high degree of conservation in 69% of specimens, we also demonstrated nucleotide differences in 8 patients (4/8 white ethnicity; 4/8 black ethnicity). Of the 8 patients, 6 patients had amino acid changes with no consistent trends in HSV variability. One patient also had a nucleotide insertion which resulted in multiple amino acid changes. Previous data have shown amino acid sequences in the N-terminal region of HSV-2 UL14 protein to be homologous to the C-terminal region of Hsp70 (Yamauchi *et al.*, 2002a). No changes were observed in this region. This

indicated the function of the UL14 protein to be preserved as an Hsp-like molecular chaperone, and have an important role in virus replication and infectivity.

The epidemiology and natural history of HSV-2 infections in HIV-1 infected individuals

Given the recent number of studies documenting a strong epidemiological association between HSV-2 and HIV-1, the seroprevalence of HSV-1 infection and its correlation with a clinical diagnosis of genital herpes was investigated in newly diagnosed individuals. In addition, we also determined HSV-2seroincidence following HIV diagnosis and its association with other STIs (Chapter 6). HSV-2 seroprevalence was high (63%) among HIV-1 infected individuals, confirming the strong positive association between the two viruses (Wald & Link, 2002; Ramjee *et al.*, 2005). Interestingly, our findings contrast previous observations on the protective effect of HSV-1 infection for HSV-2 (Gopal *et al.*, 2000; Mertz *et al.*, 1985), however others have found no evidence of this (Brown *et al.*, 1997; Langenberg *et al.*, 1999).

HSV-2 seroprevalence increased with age, and was significantly associated with female gender, heterosexual risk group and black ethnicity. This is consistent with previous data which have shown women to have a higher risk of HSV-2 infection than men (Langenberg *et al.*, 1999; Ross *et al.*, 1993; Fleming *et al.*, 1997; Vyse *et al.*, 2000; Cowan *et al.*, 1994). However, the relationship between HSV-2 seropositivity and sexual orientation is not well understood. This is due to the strong

association between gender and sexual orientation and the paucity of data comparing homosexual and heterosexual males. The strong association between black ethnicity and HSV-2 seropositivity found in our study has been reflected in other studies (Armstrong *et al.*, 2001; Gottlieb *et al.*, 2004; Mbopi-Keou *et al.*, 2000; Siegel *et al.*, 1992; Breinig *et al.*, 1990) but had not been previously demonstrated in HIV-infected patients. Elevated HSV-2 rates in HIV-1 infected patients may have major consequences for HIV-1 acquisition and transmission (Andréoletti *et al.*, 2005). In our study, HSV-2 infections continued to occur after a diagnosis of genital herpes. Of the 123 patients monitored prospectively and 12 patients (10%) seroconverted to HSV-2. HSV-2 seroconversion was strongly associated with a new diagnosis of HPV infection, gonorrhoea, hepatitis A or B and scabies and therefore was a marker of high risk sexual behaviour.

Genital herpes was significantly under-diagnosed in a large proportion of our HSV-2 seropositive HIV infected study population. Only 22% of HSV-2 seropositive patients received a clinical diagnosis of genital herpes and a fraction of those had a positive swab by virus culture. Similar findings have been reported elsewhere (Koutsky *et al.*, 1992). The presence of atypical genital lesions, asymptomatic viral shedding and poor sensitivity of virus culture could all have contributed to the lack of diagnosis. More importantly, HSV-2 seropositive patients diagnosed after 1997 were four times less likely to receive a clinical diagnosis of genital herpes than those diagnosed in the pre-HAART era. These observations suggested HAART to have a positive impact on the clinical expression of genital HSV-2 infection. Similar findings have been reported

elsewhere which showed HIV-1 positive persons on HAART reported significantly fewer days with HSV lesions than untreated subjects (Posavad *et al.*, 2004). CD4 cell counts have also shown to correlate with the clinical expression of genital herpes (Van Benthem *et al.*, 2001).

The impact of HAART on HSV-specific T-cell mediated immune responses

IFN-γ T-cell mediated immune responses in HIV-infected persons were investigated using an ELISPOT method (Chapter 7). Four different populations were studied representing different phases of the infection. These included LTNP/LTSP, newly diagnosed patients, patients on HAART and patients starting first line HAART. The strongest HSV-specific IFN-γ response was measured in LTNP/LTSP patients who had responses comparable to those of healthy HSV seropositive donors. Restoration of CD4 cell numbers was evident among patients on HAART and was accompanied by a parallel increase in HSV-specific IFN-γ responses. Overall responses in all patient groups correlated well with CD4 counts. A threshold of approximately 450 cells/μl predicted immune-reconstitution to levels similar to those in healthy donors. A larger proportion of newly diagnosed patients (67%) also received a clinical diagnosis of HSV disease compared to HAART treated individuals (23%). This observation is in agreement with our previous findings (Chapter 6) and form the basis for a future study aimed at assessing the dynamics of HSV-specific CD8 T lymphocytes in patients starting first-line HAART.

9. Chapter 9. REFERENCES

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